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Design and synthesis of new molecular scaffolds and their application as enzyme inhibitors

Ph.D. thesis of

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Chapter 1

INTRODUCTION

1.1 Peptidomimetics

The advent of genomics, molecular biology and structural biology with high-throughput X-ray crystallography and high field NMR, provided many opportunities for structural and functional analysis of a wide array of biologically active peptides. The critical roles that proteins play at all levels of biological regulation have opened a large potential for therapeutic intervention with recombinant proteins. However, the use of peptides as therapeutics is limited due to several factors, including poor bioavailability, rapid proteolytic degradation and clearance, low scale up feasibility, weak selectivity and high production cost.

Besides all these drawbacks, biomedical research is constantly oriented towards the development of new therapeutics based on peptides and proteins, by introducing specific modifications and maintaining the features responsible for biological activity.[1]

A *peptidomimetic* compound may be defined as a substance having a secondary structure and other structural features similar to native peptide such that it binds to enzymes or receptors with higher affinity than the starting peptide.

Peptidomimetics are much more selective and efficient than native peptides, thus resulting in less side effects, greater oral bioavailability and a prolonged biological activity due to lowered enzymatic degradation.[2]

The knowledge of the structure-activity relationship or the three dimensional structure of bioactive conformations is a promising route to rapidly achieve the best compound without generating an enormous number of compounds with poor biological activity.

Several peptidomimetics are reported in literature, a clear example is the thyrotropin-releasing hormone (TRH) mimetic, based on a cyclohexane scaffold. The TRH is an interesting tripeptide for its potential to treat cognitive disorders, including those associated with Alzheimer's disease. The mimetic consists in a 1,3,5-*cis*-trisubstituted cyclohexane framework which replaces the peptide backbone and bears all the pharmacophoric groups with the correct orientation for activity. Moreover, by conferring additional rigidity to the

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[1] a) Giannis, A.; Kolter, T. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1244-1267; b) Gante, J. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1699-1720; c) Adessi, C.; Soto, C. *Curr. Med. Chem.* **2002**, *9*, 963-978

[2] Olson, G. L.; Bolin, D. R.; Bonner, M. P.; Bös, M.; Cook, C. M.; Fry, D. C.; Graves, B. J.; Hatada, M.; Hill, D. E.; Kahn, M.; Madison, V. S.; Rusiecki, V. K.; Sarabu, R.; Sepinwall, J.; Vincent, G. P.; Voss, M. E. *J. Med. Chem.* **1993**, *36*, 3039-3049

system, cyclohexane determines an increase of biological activity, though side chains rotation is still allowed.[2] (Fig. 1)

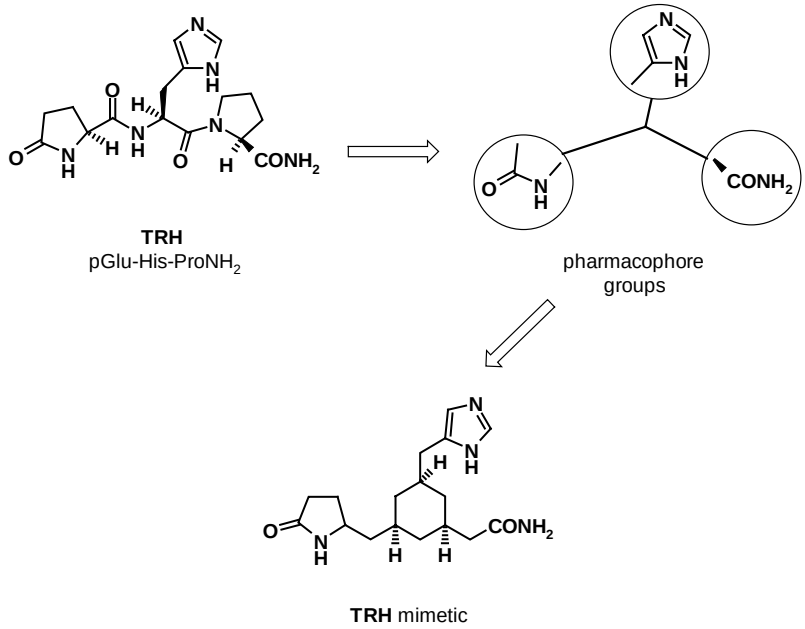

Figure **1**. Rational approach towards the development of a peptidomimetic of TRH

1.2 Bicycle molecular scaffolds (BTAa): a class of conformationally constrained dipeptide isosteres

In 1999, our research group reported on a new class of bicycle compounds based on 6,8-dioxa-3-azabicyclo[3.2.1]octane structure. Such scaffolds, named **BTAa** (**B**icycles from **T**artaric acid and **A**mino **a**cids), derived from the combination of tartaric acids and amino acid derivatives.[3] (Fig. **2**)

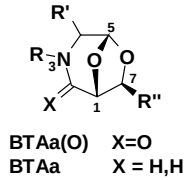

Figure **2**. BTAa structure

The main feature displayed by these molecules is the possibility of the rigid skeleton to act as a dipeptide isostere, as every functional group present on the native peptide is found at the isosteric position in the scaffold. (Fig. **3**)

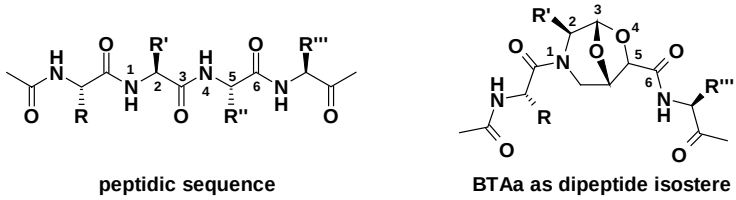

Figure **3**. BTAa scaffold acting as dipeptide isostere

Moreover, they satisfy all the requirements needed for the development of a peptidomimetic: their synthesis includes only few steps starting from commercially available enantiopure precursors and a stereochemistry control can be accomplished in each step of the synthesis; a high number of positions can be functionalised leading to molecular diversity; finally, all functional groups are well-suited for Solid Phase Peptide Synthesis, and the presence of low number of energetically permitted conformations is consistent with the ability to induce constraints in a peptide framework.

First generation of BTAa compounds was accomplished according to the retrosynthetic scheme as in Figure **4**. The combination of tartaric acid and α-amino aldehyde derivatives

[3] a) Guarna, A.; Guidi, A.; Machetti, F.; Menchi, G.; Occhiato, E. G.; Scarpi, D.; Sisi, S.; Trabocchi, A. *J. Org. Chem.* **1999**, *1*, 1407-1409; b) Trabocchi, A.; Menchi, G.; Guarna, F.; Machetti, F.; Scarpi, D.; Guarna, A. *Synlett* **2006**, 331-353

generates a scaffold with an amide group, which can be successively transformed into a bicycle amino ester or amino acid by selective reduction of the lactam moiety.

In particular, a given configuration of the stereogenic centre of amino aldehyde is obtained from the corresponding amino acid, and the choice of tartaric acid isomer, either D-, L- or meso, contributes to the overall stereochemical set of BTAa compounds.

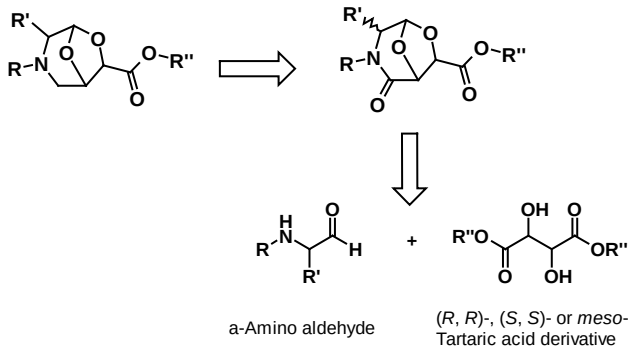

Figure **4**. Retrosynthesis of BTAa from α-amino aldehyde and tartaric acid derivative

Further experiments allowed to expand the choice of building blocks from the chiral pool in order to broaden the class of BTAa scaffolds. Sugar derivatives and amino ketones were successfully applied to give access to compounds with added diversity relatively to stereochemistry and functional group variety.[3b]

As dipeptide isosteres, BTAas are compounds with potential biological activity and their use as central core of pharmaceutical targets is convenient as a high number of positions can be functionalised leading to several molecular libraries.

The limited flexibility of the skeleton may correctly direct the pharmacophore side chains and it may be useful also for computational analysis. In addition, fair solubility and low molecular weight validate these scaffolds as potential drug candidates.

1.3 Proteases

Proteases are enzymes that catalyse the breakdown of proteins by hydrolysis of peptide bonds. Approximately 2% of the genes in most organisms are proteases, second in number only to transcription factors, and they are involved in almost every physiological process even using different catalytic mechanisms for substrate hydrolysis. [4]

The main function of proteases was long considered to be protein degradation relevant to food digestion and intracellular protein turnover, however, it was discovered that precise cleavage of proteins by proteases leads to a very subtle means of regulation.

Therefore, proteases are involved in the control of a large number of key physiological processes such as cell-cycle progression, cell proliferation and cell death, DNA replication, tissue remodelling, haemostasis, wound healing and the immune response. This leads to understand why proteases are such important drug targets, and the efforts to validate them as targets and identify or design efficient drugs against them.

Proteases can be subdivided into two major groups: exopeptidases and endopeptidases. The former progressively hydrolyses peptides or proteins starting from the amino acids at their termini, producing a mixture of amino acids. The latter hydrolyses an internal peptide bond resulting in the formation of two or more peptides as reaction products. They usually recognize specific sequences or amino acids.

Most endopeptidases are classically subdivided into 4 classes, depending on the chemical groups taking part in the hydrolytic reaction: 1) serine proteases, 2) cysteine proteases, 3) metalloproteases and 4) aspartic proteases.

The first two classes are characterized by a so called “catalytic triad”, consisting of serine, histidine and aspartate in serine proteases and of cysteine, histidine and aspartate in cysteine proteases. Metalloproteases are characterized by the direct involvement of a metal ion, usually zinc, in the catalytic process and they do not form covalent enzyme-substrate intermediates. Aspartic proteases are characterized by having in the catalytic site two aspartate residues linked to a water molecule by hydrogen bonds.

The research activity developed during my PhD in Structural Biology is focussed on two main branches related to the research of new potential selective inhibitors of

[4] a) Puente, X.S.; Sanchez, L. M.; Overall, C. M.; Lopez-Otin, C.; *Nature Rev. Genet.* **2003**, *4*, 544-558; b) Boris Turk *Nature rev. Drug Discovery* **2006**, *5*, 785-799

Metalloelastase (MMP-12), belonging to the Matrix Metalloproteases family, and of *Candida Albicans* Secreted Aspartyl Protease 2 (Sap2).

1.4 Matrix Metalloproteases

Matrix metalloproteinases (MMPs) are a family of zinc dependent, calcium containing, endopeptidases involved in extracellular matrix degradation and, consequently, they play a crucial role in physiological processes such as tissue remodelling and healing of wounds.[5] As the upregulation of MMPs is involved in many inflammatory, malignant and degenerative diseases,[6] attempts to design and develop inhibitors that may modulate their regulation have become of great interest.[7]

Although the number and nature of subunits can differ, MMP catalytic domain structures are highly similar, with a sequence identity of 50%-60%. The catalytic domain of each enzyme possesses an overall spherical topology characterized by a twisted five-stranded β-sheet, containing four parallel and one antiparallel strand, and three long α-helices. The conserved active-site sequence motif HEXXHXXGXXH coordinates the catalytic zinc(II) ion and contains a glutamic acid residue which facilitates the catalysis.[7a]

Two zinc atoms, one playing a catalytic and the other a structural role, are present in the MMP catalytic domain. The catalytic zinc ion coordinates with the three histidine residues contained within the sequence of the catalytic domain.

The enzyme latency is given by a thiol group of the cysteine residue within the propeptide which coordinates to the catalytic zinc atom. This interaction must be broken by proteolytic cleavage of the amino-terminal domain, or by conformational modification, before the metalloproteinase can degrade matrix proteins.

Once activated, MMPs are subject to inhibition by endogenous proteinase inhibitors such as the family of Tissue Inhibitors of MetalloProteinases, TIMPs. These negative regulatory controls are clearly important for a family of enzymes with such destructive potential. However, it appears that these controls do not always operate as they should, leading to a wrong expression of MMP activity which constitutes part of the pathogenic mechanism in several diseases.

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[5] Visse, R.; Nagase, H. *Circ. Res.* **2003**, *92*, 827-839

[6] Mandal, M.; Mandal, A.; Das, S.; Chakraborti, T.; Chakraborti, S. *Mol. Cell. Biochem.* **2003**, *252*, 305-329

[7] a) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. *J. Chem. Rev.* **1999**, *99*, 2735-2776; b) Skiles, J. W.; Gonnella, N. C.; Jeng, A. Y. *Curr. Med.Chem.* **2004**, *11*, 2911-2977; c) Fisher, J. F.; Mobashery, S. *Cancer Metastasis Rev.* **2006**, *25*, 115-136

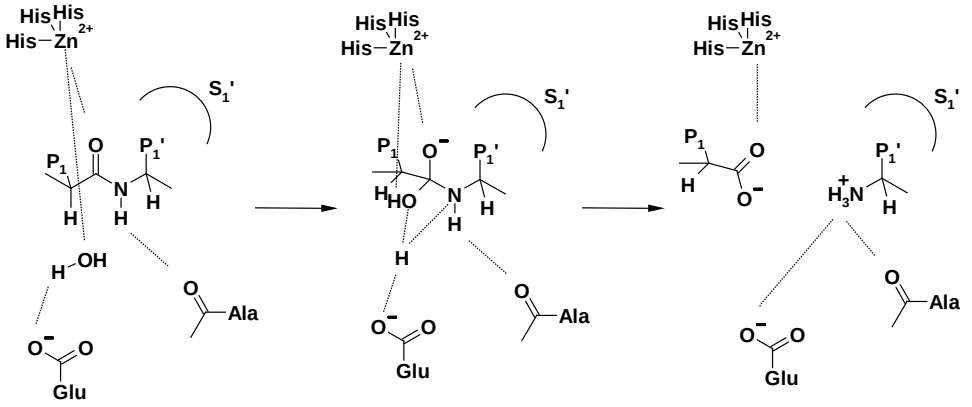

Figure **5**. MMP proteolysis reaction mechanism

The reaction mechanism for proteolysis by MMPs is depicted in Figure **5**. It is proposed that the scissile amide carbonyl coordinates to the active-site zinc(II) ion. This carbonyl is attacked by a water molecule that is both hydrogen bonded to a conserved glutamic acid and coordinated to the zinc ion.

During the proteolytic process the positively charged zinc(II) ion helps to stabilize negative charge at the oxygen of the scissile amide and a conserved alanine residue helps to stabilize positive charge at the nitrogen of the scissile amide.[7a]

From X-ray crystallographic analysis and homology modelling the MMPs may be classified as falling into two broad structural classes dependent on the depth of the S1' pocket. This "selectivity pocket" is relatively deep for the majority of the enzymes (e.g., MMP-2, MMP-8, MMP-9, MMP-12, MMP-13, etc.), but for certain enzymes (e.g., MMP-1, MMP-7, and MMP-11) it is partially or completely occluded due to an increase in the size of the side chain of the amino acids that form the pocket.

On the contrary, the other pockets of the binding site, even if they are not similar, show limited opportunities for a tight and selective binding, because they are either shallow or solvent exposed.

Over the last twenty years, many efforts were oriented towards the search of potent MMP inhibitors (MMPIs) and the most drug candidates developed to date achieves a tight binding via extensive van der Waals contacts within the largely hydrophobic interior of the S1’ pocket and by strong electrostatic interactions with the catalytic zinc ion.

Although the big effort of the scientific committee for the search of potent MMPIs, unfortunately, clinical trials on drug candidates did not show significant benefits and to date, no inhibitor of an MMP catalytic domain has become a drug.

Such insuccess may be due to the fact that the inhibitor concentration reached *in vivo* is insufficient to inhibit target enzymes in the tissue or that non-target enzymes are inhibited.[5] Since the first attempts to MMP inhibition, hydroxamic acid was the preferred zinc-chelating group, even if some authors report that it is fast metabolised *in vivo*[8] and it contributes to increase toxicity due to its low metal binding selectivity.[9]

Batimastat, a peptidomimetic hydroxamic inhibitor is an early example of MMPIs, which was extensively tested in preclinical studies. While being a potent inhibitor of MMPs with broad specificity, it is relatively insoluble. Modification of Batimastat led to the synthesis of an orally bioavailable derivative with similar inhibitory features, Marimastat, that was more suitable for clinical studies. While it was not evident in preclinical studies, both of these inhibitors when tested in the clinic promoted musculoskeletal pain as side effect.[10]

The problem of selectivity is further complicated by the presence of ADAM (A Disintegrin And Metalloproteinase) family. These membrane bound enzymes act as sheddases, liberating membrane bound cytokines, cell surface receptors, and growth factors as physiologically active soluble proteins. The catalytic site of ADAM enzymes also manifest the MMP signature sequence HEXXHXXGXXH, and small molecules that inhibit MMPs frequently also inhibit ADAM family members. It was also suggested that musculoskeletal pain associated with broad-spectrum MMP inhibitors may be due to simultaneous inhibition of ADAM proteins. This makes selectivity within and between the families even more important.

Hydroxamate replacements such as thiols, phosphonates, thiadiazoles, thiadiazines, barbituric acids, and hydantoins were investigated. Most of them are affected by weaker affinity, reduced absorption rates, or toxicity; as a result, the search for improved, novel zinc binding groups is a continuous challenge.[7c, 11]

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[8] a) Summers, J. B.; Gunn, B. P.; Mazdiyasni, H.; Goetz, A. M.; Young, P. R.; Bouska, J. B.; Dyer, R. D.; Brooks, D. W.; Carter, G. W. *J. Med. Chem.* **1987**, *30*, 2121-2126; b) Michaelides, M. R.; Dellaria, J. F.; Gong, J.; Holms, J. H.; Bouska, J. J.; Stacey, J.; Wada, C. K.; Heyman, H. R.; Curtin, M. L.; Guo, Y.; Goodfellow, C. L.; Elmore, I. B.; Albert, D. H.; Magoc, T. J.; Marcotte, P. A.; Morgan, D. W.; Davidsen, S. K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1553-1556

[9] Breuer, E.; Frant, J.; Reich, R. *Expert Opin. Ther. Patents* **2005**, *15*, 253-269

[10] Pavlaki, M.; Zucker, S. *Cancer Metastasis Rev.* **2003**, *22*, 177-203

[11] a) Puerta, D. T.; Lewis, J. A.; Cohen, S. M. *J. Am. Chem. Soc.* **2004**, *126*, 8388-8389; b) Wasserman, Z. R. *Chem. Biol.* **2005**, *12*, 143-144

The inhibitors designed up to date usually bind the active site on the catalytic domain providing nanomolar dissociation constants.[7ab, 12] However, several inhibitors exhibit a poor selectivity as a consequence of the high structural similarity among the members of the MMP family.[13] Even if the low specificity does not prevent the use *in vivo*, it raises a lot of side effects and it also limits the dose that may be daily administered.[10, 14] As a consequence, the search of new potent and selective MMP inhibitors able to get through the clinical trials, still represents an important pharmaceutical target.

[12] Fisher, J. F.; Mobashery, S. *Cancer Metastasis Rev.* **2006**, *25*, 115-136

[13] Lukacova, V.; Zhang, Y. F.; Mackov, M.; Baricic, P.; Raha, S.; Calvo, J. A.; Balaz, S. *J. Biol. Chem.* **2004**, *279*, 14194-14200

[14] Coussens, L. M.; Fingleton, B.; Matrisian, L. M. *Science* **2002**, *295*, 2387-2392

1.5 Aspartyl Proteases

Aspartyl proteases are a family of endonucleases which is widely distributed in many organisms and tissues with different physiological and functional properties.

Aspartic proteinases contain two aspartyl residues at the active site, one protonated and the other not, which work together as general acid-base catalysis. A water molecule bound between the two aspartates is believed to be the nucleophile for the amide bond hydrolysis and it is activated by the deprotonated catalytic aspartic acid residue. The nucleophilic attack by the water's oxygen to the carbonyl generates a tetrahedral intermediate whose breakdown results in the formation of amide hydrolysis products.[15]

To catalyse peptide hydrolysis, the two aspartic residues must be close enough in the tertiary structure of the molecule. They are quite far from each other in the primary structure of most aspartic proteases and the bilobed three dimensional folding allows them to be in sufficient vicinity in the catalytic site.

Most of the aspartic proteinases belong to the pepsin family, including digestive enzymes such as pepsin and chimotripsin as well as lysosomal cathepsins D and processing enzymes such as renin and certain fungal proteases (the *Candida Albicans* saps, penicillopepsin etc). A second family comprises viral proteinases such as the HIV also called retropepsin.

The active site of aspartic proteases does not in general contain groups that are sufficiently nucleophilic to be chemically modified by a selective irreversible inhibitor. Thus, most of the aspartic protease inhibitors developed to date binds to their target enzyme through non covalent interactions. These compounds are therefore reversible inhibitors and an effective inhibition results when the enzyme shows higher affinity for the inhibitor than for its natural substrate.[16]

It was proposed that stable structures which resemble the transition state of the enzyme catalysed reaction should bind the enzyme more tightly than the substrate. As a consequence, a very successful approach for the design of efficient aspartyl protease inhibitors is based on the incorporation of a transition state isostere into a peptidomimetic structure.

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[15] a) Kostka, V., Ed. *Aspartic Proteinases and Their Inhibitors* Walter de Gruyter: Berlin, **1985**; b) Chitpinityol, C.; Crabbe, M. J. C. *Food Chem.* **1998**, *61*, 395-418

[16] Tacconelli, E.; Savarino, A.; De Bernardis, F.; Cauda, R.; Cassone, A. *Curr. Med. Chem.* **2004**, *4*, 49-59

Candida Albicans is an opportunistic fungal pathogen that causes severe systemic infections especially in immunodeficient individuals.

There is a limited number of antifungal agents available[17] and the need for new drugs against *C. Albicans* is escalating due to both the widespread occurrence of mucosal infections caused by *Candida*, and the development of resistance against available drugs.[18]

Although the ability to cause disease is likely a complex process involving multiple interactions between *Candida* and the host, Secreted Aspartyl Proteases (Saps) activity appears to be the virulence factor and therefore offers a potential target for drug intervention in infections.

The *Candida* strains express at least eight distinct genes (Sap 1-8) during the course of the same disease but to different stages of infection, indicating that the different Saps have different functions;[19] Sap2 is one of the most expressed enzymes implicated in host invasion and its crystal structure is known and it is of major utility for structure designed inhibitors.[20]

[17] Rogers, T. R. *Curr. Opin. Crit. Care* **2001**, *7*, 238-241

[18] White, T. C.; Marr, K. A.; Bowden, R. A. *Clin. Microbiol. Rev.* **1998**, *11*, 382-402

[19] Schaller, M.; Schackert, C.; Korting, H. C.; Januschke, E.; Hube, B. *J. Invest. Dermatol.* **2000**, *114*, 712–717

[20] Cutfield, S. M.; Dodson, E. J.; Anderson, B. F.; Moody, P. C. E.; Marshall, C. J.; Sullivan, P. A.; Cutfield, J. F. *Structure*, **1995**, *3*, 1261-1271

1.6 Aims of the research project

In this PhD thesis new aspects are described about the design and synthesis of new molecular scaffolds having a suitable structure for acting as potential selective inhibitors of Metalloelastase (MMP-12) or of *Candida Albicans* Sap2.
Moreover, since the continuous interest towards innovative hydroxamate bioisosteres and the various potential applications of N-hydroxyurea derivatives in biological systems, a further purpose of this study consists in the investigation on new synthetic procedures on solid support which may allow the easily achievement of a large collection of different N-hydroxyurea molecules.

Thus, it will be reported about:

- The design and synthesis of BTAa derivatives showing a fair inhibition potency towards Metalloelastase and some selectivity for different MMPs, through a combined use of *in silico* screening, experimental determination of binding affinity by ^{15}N-^{1}H HSQC NMR experiments and X-ray structure analysis.
- The development of a new and efficient method for the synthesis of a novel proline mimetic based on the 6,8-dioxa-3-azabicyclo[3.2.1]octane scaffold.
- The investigation towards a new Solid Phase Organic Synthesis method to convert Fmoc-protected amino acids into O-protected N-hydroxyureas.
- The research of new potential inhibitors of Sap2**,** a major secreted aspartic proteinase from *Candida Albicans*, through two different and parallel pathways consisting in a random screening and in a rational drug design.

Chapter 2

RESULTS and DISCUSSIONS

2.1 BTAa molecular scaffolds as potential inhibitors of Macrophage Metalloelastase

Human macrophage metalloelastase (MMP-12) is a member of the MMP family which is very active against a broad range of extra cellular matrix components including elastin, fibronectin, laminin, entactin, proteoglycans and collagen IV.[21, 22]

Being able to degrade elastin, the major elastic fiber component providing resilience to tissues, MMP-12 is involved in the progressive structural damage associated with emphysema and other forms of chronic obstructive pulmonary disease (COPD).[23]

Although several drug candidates showing nanomolar dissociation constants are reported in literature, the search of new potential inhibitors still represents an important pharmaceutical target to reach better selectivity and avoid side effects.

The crystal structure analysis of the catalytic domain of human recombinant MMP-12 complexed with the broad spectrum inhibitor Batimastat (BB-94) reveals an overall fold similar to that of other MMPs.[24]

However, the characteristic S1' subsite is a continuous tube rather than a pocket, in which the specific Thr215 replaces a valine residue, otherwise highly conserved in almost all other MMPs, leading to a more polar interior of this subsite.

The availability of high-resolution structure of the MMP-12 catalytic domain[24, 25] provided the possibility to employ a structure-based approach to design and screen a virtual library with the aim to find out new specific inhibitors for emphysema therapy.

We reasoned that potential MMP-12 inhibitors could be based on the versatile BTAa scaffold. Indeed, the high degree of molecular diversifications together with a proper spatial orientation of the pharmacophoric groups may be crucial for designing truly specific inhibitors.

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[21] Belaaouaj, A.; Shipley, J. M.; Kobayashi, D. K.; Zimonjic, D. B.; Popescu, N.; Silverman, G. A.; Shapiro, S. D. *J. Biol. Chem.* **1995**, *270*, 14568-14575

[22] Mecham, R. P.; Broekelmann, T. J.; Fliszar, C. J.; Shapiro, S. D.; Welgus, H. G.; Senior, R. M. *J. Biol. Chem.* **1997**, *272*, 18071-18076

[23] Hautamaki, R. D.; Kobayashi, D. K.; Senior, R. M.; Shapiro, R. S. *Science* **1997**, *277*, 2002-2004

[24] Lang, R.; Kocourek, A.; Braun, M.; Tschesche, H.; Huber, R.; Bode, W.; Maskos, K. *J. Mol. Biol.* **2001**, *312*, 731-742

[25] Bertini, I.; Calderone, V.; Cosenza, M.; Fragai, M.; Lee, Y.-M.; Luchinat, C.; Mangani, S.; Terni, B.; Turano, P. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5334-5339

Firstly, in order to check if the scaffold could well accommodate in the MMP-12 catalytic domain and to find the best orientation for the flanking units, we decorated BTAa compounds with the most used pharmacophoric groups for MMP inhibition: the hydroxamate as ZBG, and the biphenylic residue to fit in the S1' pocket.[26]

Once demonstrated that the molecules provided by docking calculations fit in the catalytic pocket of MMP-12, we started the long challenging route of chemical modification of the scaffold to get not only an improved binding, but also better selectivity and pharmacokinetic properties.

A biphenylic moiety and a hydroxamic (or carboxylic) acid were chosen as good substituents to be introduced in different position of BTAa scaffolds and hundreds of structures were so generated and tested *in silico* for their affinity to the catalytic domain of MMP-12.

Even if the binding energy provided by docking programs does not reflect exactly the real binding affinity, its value allows to score the docking results in order to identify a class of potential ligands.[27]

Moreover, the experimental assessment of the affinity of few selected molecules outlined by docking results gives the chance to correlate binding energies and binding constants improving the prediction reliability.

The hits with the highest score were selected and, among them, compounds **1**-**4** were synthesized due to their higher feasibility.[28] In this class of molecules the scaffold supports the biphenylic moiety in front of the ZBG.

The Autodock binding model predicted a deep insertion of the biphenyl group into the S1' cavity though the coordination geometry of the ZBG appeared not to be optimized.

Dissociation constants (K_D) of compounds **1**–**4** were calculated through ^{15}N–^{1}H HSQC experiments.[29] In addition, compounds **7**–**10**, already present in our chemical library, were

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[26] Hajduk, P. J.; Sheppard, G.; Nettesheim, D. G.; Olejniczak, E. T.; Shuker, S. B.; Meadows, R. P.; Steinman, D. H.; Carrera, G. M.; Marcotte, P. A.; Severin, J.; Walter, K.; Smith, H.; Gubbins, E.; Simmer, R.; Holzman, T. F.; Morgan, D. W.; Davidsen, S. K.; Summers, J. B.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 5818-5827

[27] Klebe, G.; Gräder, U.; Grünemberg, S.; Krämer, O.; Gohlke, O.; *Understanding Receptor–Ligand Interaction as a Prerequisite for Virtual Screening. In Virtual Screening for Bioactive Molecules*, H. J. Böhm, G. Schneider, Eds.; Wiley–WCH, Weinheim, Germany, **2000**; pp 207-227

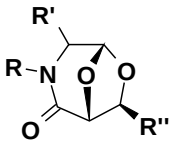
[28] Mannino, C.; Nievo, M.; Machetti, F.; Papakyriakou, A.; Calderone, V.; Fragai, M.; Guarna, A. *Bioorg. Med. Chem.* **2006**, *14*, 7392-7403

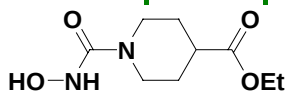
submitted to the same experiments in order to compare the results and support the docking model.

Moreover, the inhibitory activity (IC_{50}) of compounds **1**–**4** was measured by enzymatic assays.[30]

The values of K_D and IC_{50} for all the tested compounds are reported in Table **1**.

Table **1**. Calculated MMP-12 binding affinity (K_D) and inhibition potency (IC_{50}).



Compound	X	R	R'	R''	MMP-12 K_D [c]	MMP-12 IC_{50} [d]
1	O	p-PhBn	H	COOH	≥ 1mM	954 μM
2	H, H	p-PhBn	H	COOH	≥ 1mM	835 μM
3	O	p-PhBn	H	CONHOH	≥500 μM	425 μM
4	H, H	p-PhBn	H	CONHOH	≥500 μM	399 μM
5	H, H	HONHCO	H	CONHp-PhBn	154 μM	149 μM
6	O	$HONHCOCH_2$	H	CONHp-PhBn	114 μM	-
7[a]	O	Bn	H	COOH	≥ 10mM	-
8[a]	H, H	Bn	H	COOH	≥ 10mM	-
9[a]	O	Bn	H	CONHOH	≥ 10mM	-
10[a]	H,H	p-PhBn	COOH	H	not detec.[b]	-
11					3.4 mM	-

[a] Compounds previously described [5, 6, 31, 32]; [b] not detectetable interaction found by NMR; [c] dissociation constants measured through ^{15}N-^{1}H HSQC NMR experiments; [d] inhibition potency measured by fluorescence enzymatic assays.

Since the identification of the amino acids involved in the binding may be crucial to prove a direct interaction with the catalytic site and, consequently, for the optimisation of the

[29] Mitton-Fry, R. M.; Anderson, E. M.; Hughes, T. R.; Lundblad, V.; Wuttke, D. S. *Science* **2002**, *296*, 145-147

[30] Knight, C. G.; Willenbrock, F.; Murphy, G. *FEBS Lett.* **1992**, *296*, 263–266

[31] Guarna, A.; Bucelli, I.; Machetti, F.; Menchi, G.; Occhiato, E. G.; Scarpi, D.; Trabocchi, A. *Tetrahedron* **2002**, *58*, 9865-9870

[32] Trabocchi, A.; Cini, N.; Menchi, G.; Guarna, A. *Tetrahedron Lett.* **2003**, *44*, 3489-3492

process, NMR-based strategies were also used to localize the binding site on the target.[33] The identification of the binding site was easily performed analysing the protein resonance chemical shifts in $^{1}H–^{15}N$ HSQC spectra, recorded either in presence or in absence of the ligand.[34]

The pattern of the shifts was similar for all ligands. Several residues forming the S1' cavity, one or more zinc binding histidines and the neighbouring amino acids are strongly influenced by the presence of this class of molecules. As example, a ribbon representation of MMP-12 is shown in Figure **6**. The NHs showing significant chemical shift perturbation upon the addition of compound **4** are highlighted. The affected amino acids nicely define the ligand binding site that is the same for all the designed compounds in agreement with the docking results. Only few differences were found among the members of this class. In particular, the NH cross-peak of His218 was only weakly shifted by lactams **1** and **3** with respect to compound **4**.

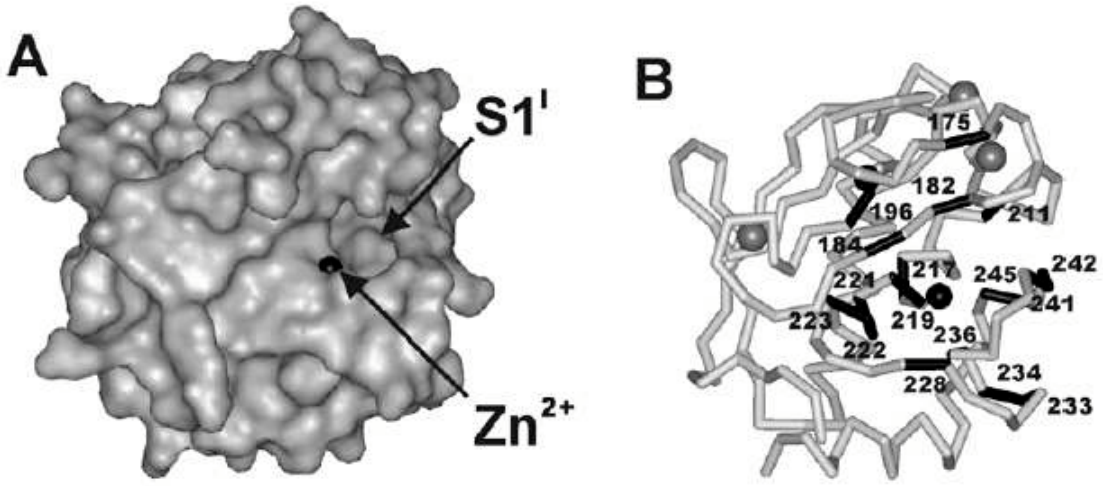


Figure **6**. A) Surface model of the MMP-12 showing the catalytic zinc and the S_1' pocket; B) Residues of the MMP-12 affected by chemical shifts perturbation upon the addition of **4**.

The analysis of the results summarized in Table **1** suggests that the bicyclic skeleton of BTAa should be compatible with the metalloelastase catalytic domain, but the activity is strongly modulated by the type and position of substituents.

[33] Pellecchia, M.; Becattini, B.; Crowell, K. J.; Fattorusso, R.; Forino, M.; Fragai, M.; Jung, D.; Mustelin, T.; Tautz, L. *Expert Opin. Ther. Targets* **2004**, *6*, 597-611

[34] Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531-1534

Since the experimental results showed first evidence of a fair inhibitory activity, we decided to focus our efforts on the design of new derivatives with improved affinity. A deeper penetration in the S1' cavity was possible moving the biphenyl group quite far from the scaffold. Due to the high reactivity of the BTAa methyl ester, it was synthetically very easy to introduce the biphenylic moiety in 7 through an amide bond. As a consequence, the best anchoring point for the ZBG resulted in the N-3 giving rise to the N-hydroxyurea **5**.

The use of the hydroxyurea group as metal chelator in MMP inhibitors has been previously reported[35] and recently validated by a X-ray structure.[36]

In order to determine the affinity of the N-hydroxyurea moiety with the MMP-12 catalytic zinc ion, the model compound **11** was tested *in silico* and synthesized. Since the metal binding capability of the N-hydroxyurea was confirmed, the new scaffold **5** was synthesized.

The $^{1}H–^{15}N$ HSQC spectra for **5** showed a pattern of shifts similar to compounds **1**–**4** indicating for all these molecules the same binding site (Fig. **7**). A different effect on the zinc binding histidine with respect to the N-hydroxyurea prototype is probably present since the shift perturbation involved mainly His222 instead of His218. The fit of $\Delta\delta$ as a function of the ligand concentration provided a K_D of 154 μM, in good agreement with the IC_{50} value of 149 μM obtained by the enzymatic assay.

[35] Michaelides, M. R.; Dellaria, J. F.; Gong, J.; Holms, J. H.; Bouska, J. J.; Stacey, J.; Wada, C. K.; Heyman, H. R.; Curtin, M. L.; Guo, Y.; Goodfellow, C. L.; Elmore, I. B.; Albert, D. H.; Magoc, T. J.; Marcotte, P. A.; Morgan, D. W.; Davidsen, S. K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1553-1556

[36] Campestre, C.; Agamennone, M.; Tortorella, P.; Prezioso, S.; Biasone, A.; Gavazzo, E.; Pochetti, G.; Mazza, F.; Hiller, O.; Tschesche, H.; Consalvi, V.; Gallina, C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 20-24

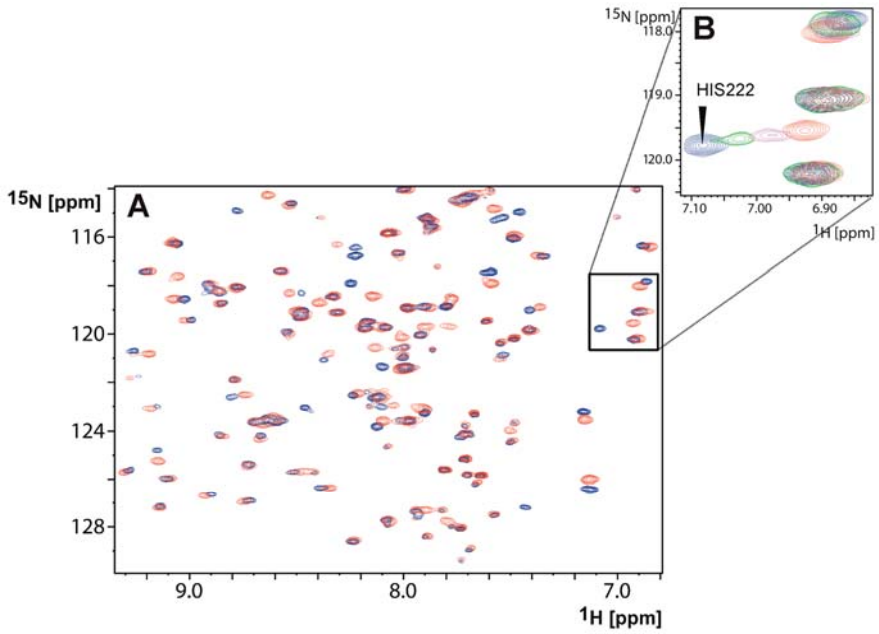


Figure **7**. A) ^{1}H-^{15}N HSQC spectrum of MMP-12 catalytic domain in absence (blue) and in presence (red) of compound **5** (500 μM); B) Expansion of ^{1}H-^{15}N HSQC spectrum showing the shift of the His222 backbone NH upon the addition of increasing concentration of compound **5**.

To probe the selectivity towards MMP-12, N-hydroxyurea **5** was also tested by fluorescence enzymatic experiments for the inhibition of collagenases (MMP-1, MMP-8, MMP-13), matrilysin 1 (MMP-7) and stromelysin 2 (MMP-10) and the corresponding data are summarized in Table 2.

Table **2.** MMP enzyme inhibition potency of compound **5** measured by enzymatic assay

Compound	**IC_{50}** (μM)					
	MMP-1	**MMP-7**	**MMP-8**	**MMP-10**	**MMP-12**	**MMP-13**
5	1180	1510	513	865	149	778

Compound **5** is a fairly selective MMP-12 inhibitor since it possesses limited activity against the other MMPs especially the MMP-1 and MMP-7. These two enzymes differ from other MMP family members since they have a relatively small S1' pocket which presumably cannot accommodate the large biaryl substituent.

A lack of activity with respect to MMP-1 is considered to be an important factor in reducing some of the side effects found in drug candidates. Several MMP inhibitors, in fact, showed a severe musculoskeletal syndrome as side effect, which seems to be linked to

an impairment of normal tissue remodelling governed either by the MMP-1 or by sheddases.

Although compound **5** exhibits an improved affinity towards MMP-12, nevertheless the micromolar value of K_D demonstrates that all the planned interactions are not optimized. The X-ray structure of the MMP-12–**5** complex (PDB code: 2HU6) provided a direct inspection on ligand–protein interactions (Fig. **8**).

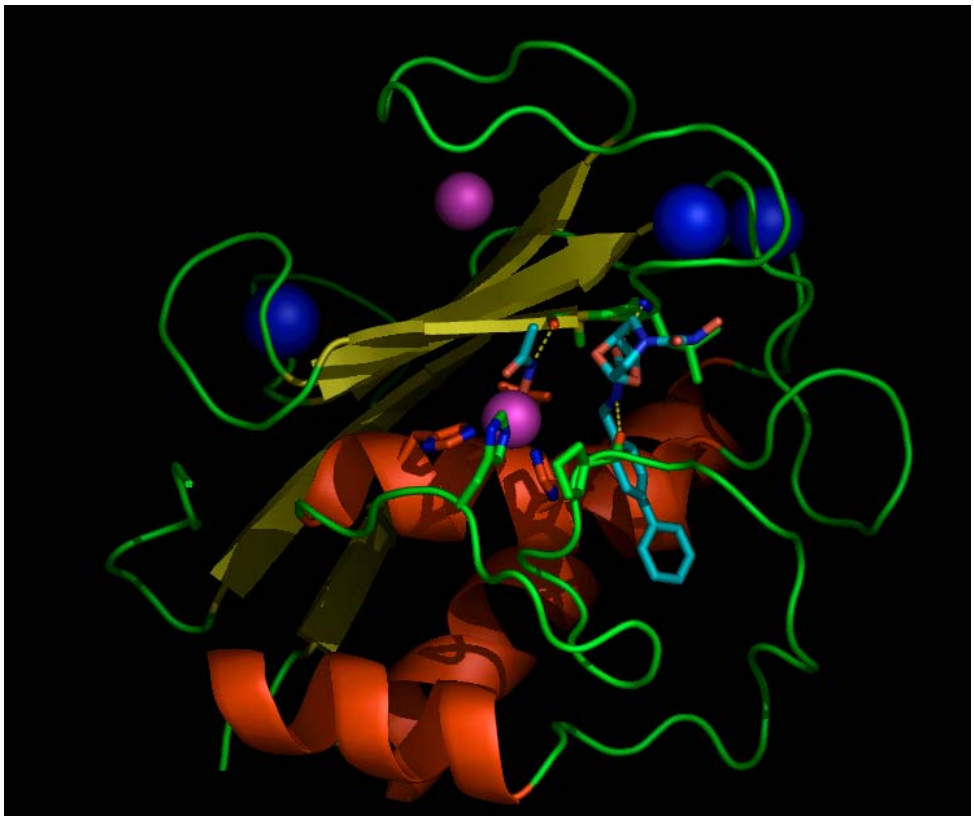


Figure **8**. Crystal structure of MMP-12-**5** adduct, the zinc ion is coordinated by AHA

As correctly predicted by the NMR studies the molecule binds the metalloelastase at the catalytic site, fitting the biaryl moiety into the S1' pocket. The electron density of the inhibitor is well defined because it is held in place by two hydrogen bonds with Pro238 and Leu181 of the enzyme backbone, as clearly shown in Figure **9**. However, the contribution of the hydroxyurea to the binding is absolutely negligible since it points towards the exterior of the active site and does not interact with the catalytic zinc ion. Noteworthy, the zinc ion is coordinated by the weak inhibitor acetohydroxamic acid (AHA) present, as stabilizer, in the crystallization buffer. This would suggest that the lack of interaction between the hydroxyurea and the catalytic zinc, evidenced in the crystal structure, is due to the competition with the high concentration of AHA.

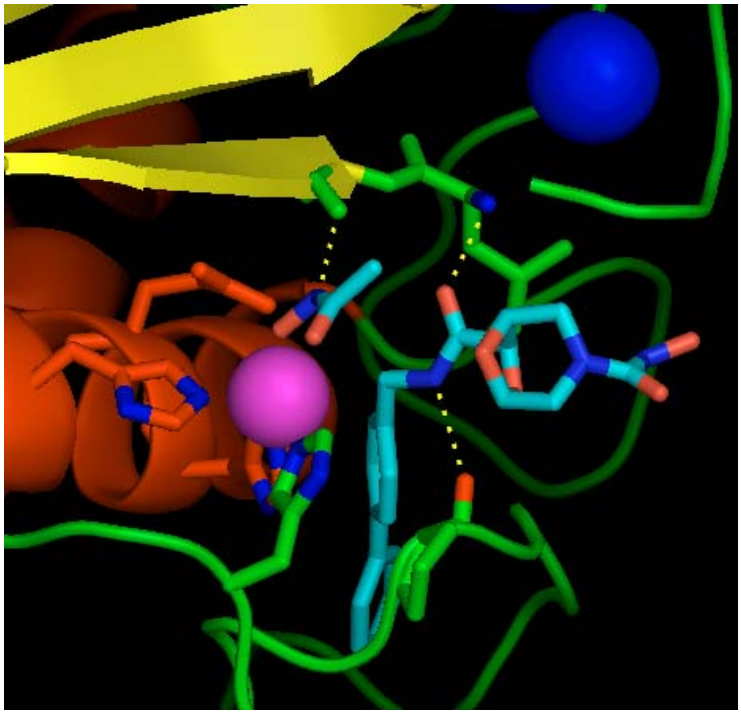


Figure **9**. Crystal structure of MMP-12-**5** adduct, particular showing the two hydrogen bonds with Pro238 and Leu181 of the enzyme backbone.

In order to investigate this point, an energy minimization of the ligand was performed after removing AHA from the PDB and blocking the biaryl group in the crystal structure conformation. The AMBER-8 minimized model indicates that the hydroxyurea function could coordinate the zinc ion, even if the hydrogen bond interactions with the zinc binding site are not well defined as in the case of AHA (Fig. **10**).

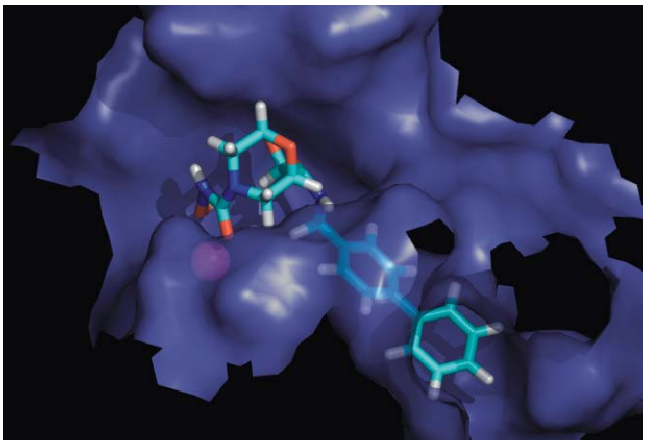
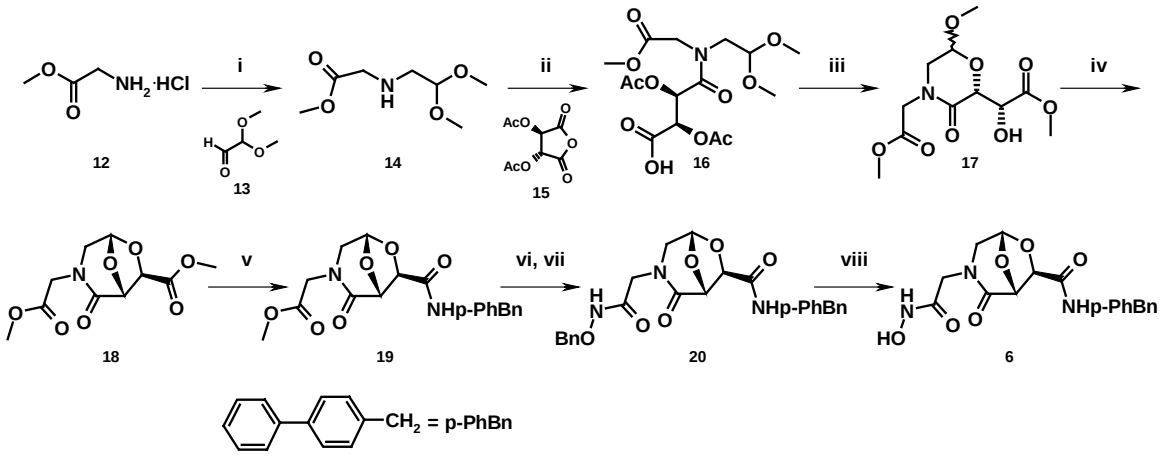


Figure **10**. AMBER-8 minimized model of MMP-12-**5** adduct.

As these data suggested that the anchoring point and/or the length of the ZBG are not optimized, a more flexible hydroxamic acid derivative (**6**) was obtained by inserting a

methylene group between the ZBG and the scaffold with the aim to promote the binding with the catalytic zinc ion (Scheme **1**).



Scheme **1**. Reagents and conditions: (i) NEt_3, MeOH, H_2, Pd/C, r.t. o/n (59%); (ii) DCM, r.t. o/n; (iii) $SOCl_2$, MeOH, 60°C, 2 h.; (iv) SiO_2/H^+, toluene, reflux, 20 min (17% over three steps); (v) H_2N-BnPh, DCM, 40°C, o/n (88%); (vi) LiOH, MeOH/THF r.t. o/n; (vii) $BnONH_2$, HOBT, DIC, DIPEA, DCM r.t. o/n (40%); (viii) H_2, Pd/C, THF, r.t. o/n

The synthesis of compound **6** was carried out following a procedure previously reported for similar compounds and it is briefly reported below.[37]

Reductive alkylation of glycine methylester with dimethoxy-acetaldehyde (**13**) afforded the secondary amine **14** which was subsequently converted into the amide **16** through a coupling reaction with tartaric acid derivative **15**[38]. Treatment of crude **16** with thionyl chloride in MeOH afforded cyclic acetal **17** which was further submitted to the acid-catalysed cyclisation to yield **18** in 17% yield over the three steps.

Thanks to the peculiar reactivity of the methyl ester in 7, amide **19** was easily synthesized in good yield by mixing the scaffold with a small excess of *p*-phenyl-benzyl amine in DCM at 40°C.

Basic hydrolysis of the methyl ester in 2 followed by a coupling reaction with O-benzyl hydroxylamine afforded the protected hydroxamic acid **20**. Mild hydrogenolytic cleavage of the protecting group over 10% Pd/C yielded the title compound **6**.

[37] Guarna, A.; Guidi, A.; Machetti, F.; Menchi, G.; Occhiato, E. G.; Scarpi, D.; Sisi, S.; Trabocchi, A. *J. Org. Chem.* **1999**, *1*, 1407-1409

[38] Lucas, H. J.; Baumgarten, W. *J. Am. Chem. Soc.* **1941**, *63*, 1653-1657

The ^{1}H-^{15}N HSQC spectra for **6** provided a K_D of 114 μM which shows a weak improvement in the activity even if the main binding contribution is still determined by the insertion of the biphenyl moiety in the S1' cavity.

2.2 Synthesis of a bicyclic proline analogue from L-Ascorbic Acid

Peptides and proteins are essential elements of organisms. While proteins have biocatalytic functions and are important components of tissues, peptides influence a multitude of physiological processes as hormones, neurotransmitters and neuromodulators.

Since bioactive peptides play an important role in the appearance and maintenance of various diseases, agents that could imitate or block their biological functions (agonist or antagonist, respectively) were considered as aid for the investigation on therapeutics.

Bioactive peptides, due to their pharmacological properties, can be employed as drugs in only a few cases. Therefore, peptidomimetics were developed with the aim to substitute peptides in their interactions with receptors; indeed, in comparison with native peptides, they show higher metabolic stability, better bioavailability and longer duration of action.

The need to replace natural amino acids in peptides with non-proteinogenic counterparts in order to obtain drug-like target molecules stimulated a great deal of biomedical research.[39]

In this respect, the field of amino acids gained large relevance in recent years, particularly with the emergence of unnatural analogues as components of molecules with therapeutic potential.[40]

Of particular interest was the research of mimetics of the only cyclic proteinogenic α-amino acid, proline, because it plays a critical role in protein folding and refolding.

As proline can exhibit *cis*-*trans* rotamerization around the amide bond, Xaa-Pro peptide bond has approximately the same energy in both *cis* and *trans* conformer, unlike other peptide amide bonds which adopt predominantly more stable *trans* conformations.

As a consequence, proline participates in nucleation of turns as well as in breaking of helices in proteins, and exerts a significant influence upon a number of biologically important processes.

Among the various approaches for mimicking peptide structures,[41] numerous mimetics and analogues of proline were developed with the aim to gain more insight into the modulation

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[39] a) Giannis, A.; Kolter, T. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1244-1267; b) Gante, J. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1699-1720; c) Olson, G.L.; Bolin, D.R.; Bonner, M.P.; B6s, M.; Cook, C.M.; Fry, D.C.; Graves, B.J.; Hatada, M.; Hill, D.E.; Kahn, M.; Madison, V.S.; Rusiecki, V.K.; Sarabu, R.; Sepinwall, J.; Vincent, G.P.; Voss, M.E. *J. Med. Chem.* **1993**, *36,* 3039.

[40] a) Sardina, F.J.; Rapoport, H. *Chem. Rev.* **1996**, *96,* 1825; b) Ohfune, Y. *Acc. Chem. Res.***1992**, *25,* 360.

[41] Hanessian, S.; McNaughton-Smith, G.; Lombart, H. G.; Lubell, W. D. *Tetrahedron* **1997**, *53*, 12789-12854

of the *cis*/*trans* isomerism of acyl-proline bonds[42] and to produce proline-like reverse turn inducers;[43] moreover, they were applied as central core of several pharmacologically active small-molecules.[44]

Since the development of the first examples of 6,8-dioxa-3-azabicyclo[3.2.1]octane-based scaffolds (BTAa, Fig. **11**), various substituents were introduced at positions 3, 4, 5, and 7,[45] whereas position 2 remained largely unexplored, occupied only by carbonyl, tiocarbonyl, or methylene groups.

During the last years, our research group explored the possibility of moving the carboxylic group from the canonical 7*exo* position in terms of stereochemical and positional shift. This challenge required the development of new efficient synthetic strategies to obtain new BTAa-based amino acids.

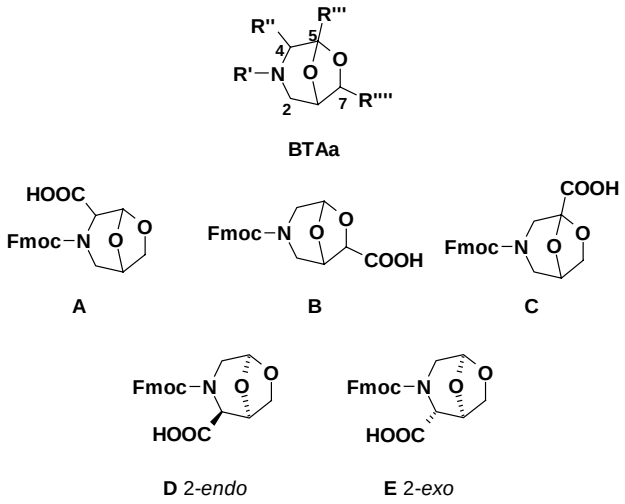

Figure **11**. General formula of BTAa scaffolds (*top*) and isomeric bicyclic amino acids **A**–**E**, with **A, D** and **E** as proline mimetics

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[42] a) Wang, Q.; Tran Huu Dau, M.-E.; Sasaki, N. A.; Potier, P. *Tetrahedron*, **2001**, *57*, 6455-6462; b) Demange, L.; Cluzeau, J.; Ménez, A.; Dugave, C. *Tetrahedron Lett.* **2001**, *42*, 651-653

[43] a) Kym, K.; Germanas, J. P. *J. Org. Chem.* **1997**, *62*, 2847-2852; b) Zhang, W. J.; Berglund, A.; Kao, J. L.-F.; Couty, J. P.; Gershengorn, M. C.; Marshall, G. R. *J. Am. Chem. Soc.* **2003**, *125*, 1221-1235

[44] a) Cheng, M.; De B.; Almstead, N. G.; Pikul, S.; Dowty, M. E.; Dietsch, C. R.; Dunaway, C. M.; Gu, F.; Hsieh, L. C.; Janusz, M. J.; Taiwo, Y. O.; Natchus, M. G. *J. Med. Chem.* **1999**, *42*, 5426-5436; b) Damour, D.; Herman, F.; Labaudinière, R.; Pantel, G.; Vuilhorgne, M. ; Mignani, S. *Tetrahedron* **1999**, *55*, 10135-10154 ; c) Dahlgren, A.; Braualt, J.; Kuaenström, I.; Nilsson, I.; Musil, D.; Samuelsson, B. *Bioorg. Med. Chem.* **2002**, *10*, 1567-1580; d) Wang, X. J.; Hart, S. A.; Xu, B.; Mason, M. D.; Goodell, J. R.; Etzkorn, F. A. *J. Org. Chem.* **2003**, *68*, 2343-2349

[45] Trabocchi, A.; Menchi, G.; Guarna, F.; Machetti, F.; Scarpi, D.; Guarna, A. *Synlett* **2006**, 331-353

A complete change of the COOH position was firstly achieved using side chain of serine as source of the carboxylic moiety. A new set of bicyclic compounds, named BGS, was then synthesized. The shift of the carboxyl moiety from position 7 to 4 allowed the generation of constrained unnatural α-amino acids showing structural features similar to proline.[46] (Fig. **11**, **A**)

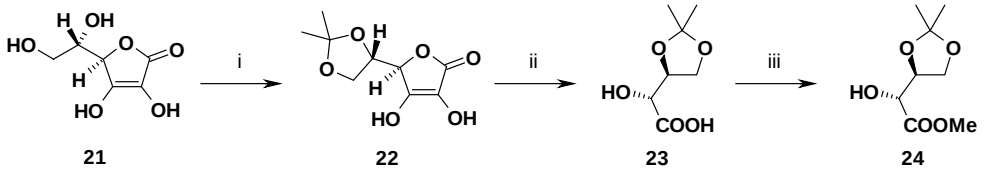
Successively, a novel procedure to obtain a new scaffold with the carboxyl function in position 5 allowed the generation of a β-amino acid isomeric to α-amino acids BGS and to γ/δ-amino acid BTG compounds.[47] (Fig. **11**, **C**)

In order to complete the collection of amino acids that the BTAa scaffold may originate, we decided to investigate a strategy for the synthesis of new α-amino acid scaffolds, mimetics of proline, in which the COOH group is shifted to the almost unexplored position 2.[48] (Fig. **11**, **D**, **E**)

We reasoned that using L-ascorbic acid as a convenient and inexpensive starting material, we could efficiently reach our goal.

L-Ascorbic acid appeared in literature as a valuable source of chiral building blocks for the preparation of enantiopure β-lactams[49] and L-hexoses[50], key components of numerous biologically active natural products.

The key intermediate L-threonic acid derivative **24** which can be efficiently obtained from L-ascorbic acid **21** by the degradation method of Isbell and Frush,[51] contains a suitably functionalised carbon skeleton for our synthesis and the proper chirality.



21 **22** **23** **24**

Scheme **2**. Reagents and conditions: i) 2,2-dimethoxypropane, pTSA, acetone, r.t. 1h; ii) $CaCO_3$, H_2O_2 35%, Pd/C, H_2O, 0°-20°C; iii) $NaHCO_3$, Me_2SO_4, H_2O, 40°C, 6h 70%

[46] Trabocchi, A.; Cini, N.; Menchi, G.; Guarna, A. *Tetrahedron Lett.* **2003**, *44*, 3489-3492

[47] Danieli, E.; Trabocchi, A.; Menchi, G.; Guarna, A. *Eur. J. Org. Chem.* **2005**, 4372-4381

[48] Lalli, C.; Trabocchi, A.; Guarna, F.; Mannino, C.; Guarna, A. *Synthesis* **2006**, *18*, 3122-3126

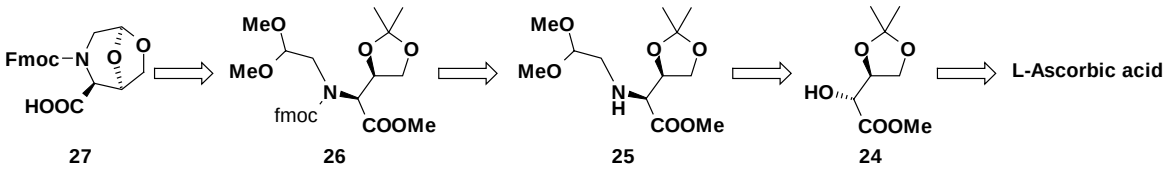
[49] Wei, C. C.; De Bernardo, S.; Tengi, J. P.; Borgese, J.; Weigele, M. *J. Org. Chem.* **1985**, *50*, 3462-3467

[50] Ermolenko, L.; Sasaki, A. *J. Org. Chem.* **2006**, *71*, 693-703

[51] Isbell, H. S.; Frush, H. L. *Carbohydr. Res.* **1979**, *72*, 301

It was obtained upon oxidation of the protected L-ascorbic acid **22** with hydrogen peroxide and successive esterification performed with dimethyl sulfate and sodium bicarbonate in water. (Scheme **2**)

The key intermediate **24** was converted to the corresponding triflate and then, upon treatment with amino acetaldehyde derivative, transformed to amino acetal **25**. Fmoc protection and successive acid cyclization performed at 25°C afforded the desired bicycle scaffold allowing a concomitant deprotection of the methyl ester functionality. (Scheme **3**)



Scheme **3**. Retrosynthesis of a bicyclic α-amino acid with an *endo*-carboxy group from L-ascorbic acid

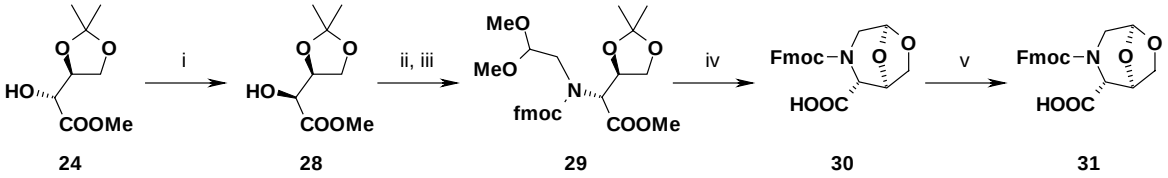
Formal inversion of the configuration at the C-2 stereocenter of compound **27** gives, through a similar synthetic pathway, the corresponding diastereomer, carrying the carboxyl group in the 2-*exo* position (Fig. **11**, compound **E**).

Chloroacetate alcoholysis in mild basic conditions, followed by hydrolysis with sodium hydrogen carbonate, permitted the inversion of configuration at the C-2 of the key intermediate **24** in good yield.[52]

Interestingly, the acid conditions applied for cyclization did not allowed, as verified in case of diastereoisomer **D**, the concomitant deprotection of the methyl ester function which had to be carried out in a further step by refluxing a solution of aqueous HCl in CH_3CN for 16 hours. (Scheme **4**)

The different behaviour of the two diastereoisomers led to the hypothesis that the facile hydrolysis to give compound **27** might occur through the urethane carbonyl group providing anchimeric assistance to the equatorial methoxycarbonyl group.

[52] a) Ermolenko, L.; Sasaki, N. A. J. Org. Chem. 2006, 71, 693-700; Saïah, M.; Bessodes, M.; Antonakis, K. *Tet. Lett.* **1992**, *33*, 4317-4320



24 28 29 30 31

Scheme **4**: Reagents and conditions: i) $ClCH_2COOH$, PPh_3, THF, 12h, then $NaHCO_3$, MeOH 49%; ii) Tf_2O, Pyridine, DCM, -10°C, 30'; iii) aminoacetaldehyde dimethyl acetal, DIPEA, DCM, 0°C, 22h; iv) FmocCl, 2,6-lutidine, dioxane, 0°C-r.t. o/n; v) TFA, 25°C, 48h; vi) HCl, CH_3CN, reflux, 16h.

In conclusion, we reported a new and efficient method for the synthesis of two proline mimetics derived from L-ascorbic acid. The use of such an inexpensive starting material is an advantage also for a potential multigram-scale organic synthesis.

The two new bicyclic proline analogues may find application in peptidomimetic research, and, in particular, are suited for solid-phase organic and peptide synthesis by the Fmoc protocol.

Since in Matrix Metallo Proteinases the catalytic zinc II ion and the selectivity pocket S1' are close to each other, it was supposed that the presence in vicinal position of the two main pharmacophore groups of a potential MMPI could promote a tight binding. Therefore, a proper decoration of proline mimetics appeared as an interesting route for the MMP-12 inhibitor research, as also evidenced by the conformationally constrained hydroxamic acids related to proline scaffold which are reported in literature as interesting compounds for MMP inhibition.[53]

Nevertheless, we reported that the BGS based α-amino acid **10** did not show, through NMR experiments, a detectable interaction with the MMP-12 catalytic domain (See Table **1**, pag. 19); hence, we decided to get more insight into this field by changing the relative spatial orientation of the two binding groups for a potential better fitting.

In this respect, further decoration of the two proline mimetics D and E will be accomplished by inserting the biphenylic moiety in place of the Fmoc-protecting group and by transforming the carboxylic acid in a better zinc binding group.

[53] Cheng, M.; De B.; Almstead, N. G.; Pikul, S.; Dowty, M. E.; Dietsch, C. R.; Dunaway, C. M.; Gu, F.; Hsieh, L. C.; Janusz, M. J.; Taiwo, Y. O.; Natchus, M. G. *J. Med. Chem.* **1999**, *42*, 5426-5436

2.3 Solid phase N-hydroxyurea synthesis: an efficient method to convert Fmoc-amino acids to O-protected-hydroxyureas

Over the last ten years, investigations towards new potential hydroxamate bioisosteres were carried out either in pharmaceutical company or in academy and, in this respect, it increased the interest on hydroxyurea derivatives as they possess metal-chelating and redox-properties.

The simplest member of this class, N-hydroxyurea (HU), currently finds clinical use as an antineoplastic agent for the treatment of leukemia and other malignant tumour diseases.

HU is able to quench the tyrosil free radical at the active site of the riboncleotide reductase, the enzyme which catalyses the conversion of the four ribonucleotides to deoxyribonucleotides for DNA synthesis.

Although the detailed mechanism of the HU action is not still thoroughly determined, this compound has been approved for the treatment of oncological diseases. It is well adsorbed and it is excreted in the urine with a biological half life of less than 8 hours. Since it inhibits DNA synthesis and has a brief duration, the drug is toxic only to cells that are actively synthesizing DNA during the period of drug exposure.[54]

As HU can selectively inhibit DNA synthesis of malignant cells, it is also useful as a treatment for sickle cell anemia[55] and it inhibits HIV-1 proviral DNA synthesis in infected cells. The antiviral effect is achieved at non toxic doses, lower than thus currently used in human therapy.[56]

Since N-hydroxyurea targets a cellular enzyme instead of a viral one, no viral mutations occur after repeated exposure to the drug and consequently it is not expected to generate high rates of escape mutants.

Moreover, N-Hydroxyurea derivatives act as inhibitors of various metal containing hydrolytic enzymes such as carbopeptidase A,[57] carbonic anhydrase,[58] matrix

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[54] Gao, W.-Y.; Johns, D. G.; Mitsuya, H. *Mol. Pharmacol.* **1994**, *46*, 767-772.

[55] Charache, S.; Terrin, M. L.; Moore, R. D.; Dover, G. J.; Barton, F. B.; Eckert, S. V.; McMahon, R. P.; Bonds, D. R. *N. Engl. J. Med.* **1995**, *322*, 1317-1322.

[56] Lori, F.; Malykh, A.; Cara, A.; Sun, D.; Weinstein, J. N.; Lisziewicz, J.; Gallo, R. C. *Science* **1994**, *266*, 801-805.

[57] Chung, S. J.; Kim, D. H. *Bioorg. Med. Chem.* **2001**, *9*, 185-189.

[58] Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2003**, *11*, 2241-2246.

metalloproteases,[59] urease[60], ribonuclease[61] and redox enzymes including lipoxygenase[62] and ribonucleotide reductase.[63]

N-hydroxyureas are interesting bioisosteres of the hydroxamate pharmacophore, which has very poor pharmacokinetic properties due to rapid hydrolysis to the corresponding, weaker inhibitor, carboxylic acid.

In the last years some research groups carried out investigations towards potential hydroxamate bioisosteres based on either retrohydroxamate or N-hydroxyurea moiety.

Abbott Laboratories outlined a family of N-hydroxyurea containing compounds as bioisosteres of the hydroxamate pharmacophore that might provide potent 5-lipoxygenase inhibitors by offering greater resistance against hydrolytic, oxidative and conjugative metabolism.[60] They also pursued a research on alternative zinc binding groups for MMP inhibitors in order to gain a better in vivo stability by exploring different kinds of hydroxyurea derivatives. [59a]

Gallina and co-workers published the first crystallographic structure of a N-hydroxyurea inhibitor bound into the active site of a matrix metalloprotease and compared the binding with the catalytic zinc ion to that of hydroxamic acid derivatives.[59b]

The interest in identifying innovative hydroxamate bioisosteres and the various potential applications of N-hydroxyurea derivatives in biological systems suggested the necessity to find new synthetic procedures able to easily achieve a large collection of different N-hydroxyurea molecules.

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[59] a) Michaelides, M. R.; Dellaria, J. F.; Gong, J.; Holms, J. H.; Bouska, J. J.; Stacey, J.; Wada, C. K.; Heyman, H. R.; Curtin, M. L.; Guo, Y.; Goodfellow, C. L.; Elmore, I. B.; Albert, D. H.; Magoc, T. J.; Marcotte, P. A.; Morgan, D. W.; and Davidsen, S. K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1553-1556. b) Campestre, C.; Agamennone, M.; Tortorella, P.; Prezioso, S.; Biasone, A.; Gavazzo, E.; Pochetti, G.; Mazza, F.; Hiller, O.; Tschesche, H.; Consalvi, V.; Gallina, C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 20-24. c) Mannino, C.; Nievo, M.; Machetti, F.; Papakyriakou, A.; Calderone, V.; Fragai, M.; Guarna, A. *Bioorg. Med. Chem.* **2006**, *14*, 7392-7403

[60] Uesato, S.; Hashimoto, Y.; Nishino, M.; Nagaoka, Y.; Kuwajima, H. *Chem. Pharm. Bull.* **2002**, *50*, 1280-1282.

[61] Higgin, J. J.; Yakovlev, G. I.; Mitkevich, V. A.; Makarov, A. A.; Raines, R. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 409-412.

[62] Stewart, A. O.; Bhatia, P. A.; Martin, J. G.; Summers, J. B.; Rodriques, K. E.; Martin, M. B.; Holms, J. H.; Moore, J. L.; Craig, R. A; Kolasa, T.; Ratajczyk, J. D.; Mazdiyasni, H.; Kerdesky, F. A. J.; DeNinno, S. L.; Maki, R. G.; Bouska, J. B.; Young, P. R.; Lanni, C.; Bell, R. L.; Carter, G. W.; and Brooks, C. D. W. *J. Med. Chem.* **1997**, *40*, 1955-1968.

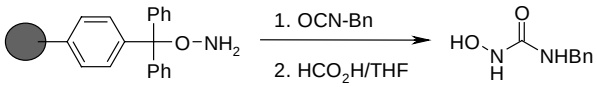
[63] Young, C. W.; Schochetman, G.; Hodas, S.; Balis, M. E. Cancer Res. 1967, 27, 535-540

Solid Phase Organic Synthesis and Combinatorial Chemistry during the last decades provided new opportunities for medicinal chemistry research by allowing the synthesis of molecular libraries.

Thus, the experience on N-hydroxyurea derivatives synthesis we reached during the research of potential MMP-12 inhibitors suggested us the possibility to develop a new SPOS method for N-hydroxyurea containing libraries.

As N-hydroxyurea libraries play an important role in the search for potential drug candidates, an efficient and practical method for the solid phase synthesis of these compounds is of considerable interest.

To our knowledge, there are only few examples in literature of solid phase method for N-hydroxyurea synthesis. Bauer and co-workers described a novel linkage for the solid phase synthesis of hydroxamic acids. In this context, they reported one example, as depicted in Scheme **5**, to evidence the chance to apply their method also to afford urea-type hydroxamic acids by treatment with isocyanates.[64]

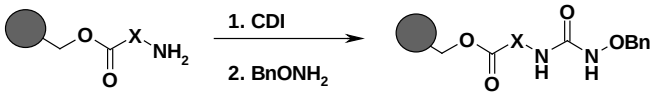


Scheme **5**. Solid phase urea-type hydroxamic acid synthesis reported by Bauer and co-workers

The use of the described linkage does not allow a direct solid phase synthesis of peptidic or peptidomimetic N-hydroxyureas as the skeleton of the desired compounds cannot be progressively built on the solid support. Moreover, diversification of the molecules is limited to the readily available isocyanates and the O-nitrogen atom cannot be further alkylated.

Thus, we developed a new easy and efficient solid phase procedure to obtain protected N-hydroxyureas starting from Fmoc-protected amines. Activation of resin bound amino acids with carbonyliimidazole (CDI), followed by treatment with O-benzylhydroxylamine, provided the desired substituted N-hydroxyurea in good yield. Hydrogenolytic cleavage of the benzyl group over 5% Pd/C easily afforded the free hydroxyurea.

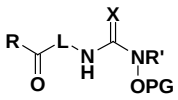
[64] Bauer, U.; Ho, W.-B.; Koskinen, A. M. P. *Tet. Lett.* **1997**, *38*, 7233-7236.



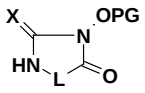
Scheme **6**. General solid phase organic procedure for O-Benzyl Hydroxyureas synthesis

The main advantage of this transformation over existing methods is its flexibility which may assure a high degree of diversification. In Table **3** are depicted the two accessible products and their potential diversifications. The L group corresponds to the main molecular structure, consisting of peptides, peptidomimetics, all kind of amino acids and, mostly, all the structures bearing, besides an amine, a functional group able to bind the solid support.

Table **3**. Summary of potential diversifications permitted by the spos method



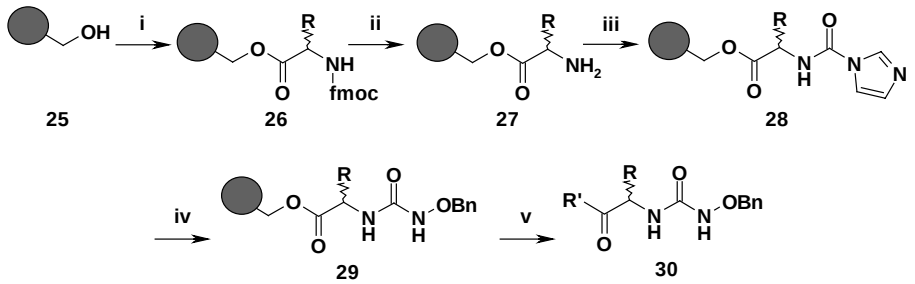
N-Hydroxyurea derivatives



N-Hydroxyhydantoine derivatives

R	**R'**	**X**	**PG**
OMe	H	O	Bn
R''NH	Bn	S	TBDMS
R''R'''N	Alkyl		H
NH_2			
OH			

The O-protected N-hydroxyureas synthesized reported in Table **4**, were obtained following the procedure depicted in Scheme **7**.

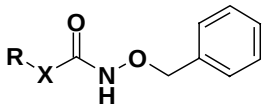


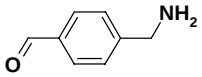
Scheme **7**. Reagents and conditions: HMBA-AM resin; (i) Amino acid, HOBT, DIC, DMAP, DMF, r.t. o/n; (ii) Piperidine 20% in DMF, r.t. 30'; (iii) CDI, DCM, 0°C - r.t. 1h; (iv) $BnONH_2$, DCM, r.t. o/n; (v) cleavage

The binding of the amino acid to the HMBA-AM resin was accomplished using HOBT, DIC and DMAP as coupling agents; after Fmoc deprotection, crude resin was treated at 0°C with a solution of CDI in DCM and the resulting mixture was allowed to reach room temperature and stirred for 1 hour. After removing the solution, and washing the resin with DCM and THF, $BnONH_2$, as a free base, in DCM was added and the suspension was shaken overnight at room temperature. (Scheme **7**)

Protected hydroxyureas were cleaved off the HMBA-AM linker with a number of different nucleophiles to yield compounds with a variety of functional groups at the C-terminus, such as esters and amides. (Table **4**)

Table **4**. Synthesized O-Benzyl-Hydroxyureas and cleavage conditions



Entry	X	R	Cleavage conditions	Yield	Purity[a]
31	Phe	EtNH	$EtNH_2$ (70% in H_2O), THF, r.t. o/n	40%	- [b]
32	PheGly	*i*BuNH	*i*$BuNH_2$, THF, H_2O cat., r.t. o/n	28%	50%
33	Val-Phe	MeO	MeOH/NH_3 (9:1), 50°C o/n	83%	- [b]
34		MeO	MeOH/NH_3 (9:1), 50°C o/n	44%	51%

a) Determined by HPLC analysis of crude product after cleavage from the resin; b) data not yet available

Although primary amines generally reacted affording the desired products with satisfactory yields, amino benzoic acid failed to react according to this method probably since its low nucleophilic character.

Also secondary amines missed the conversion to hydroxyureas through the procedure described. Firstly, a steric hindrance issue was supposed to prevent the proline from

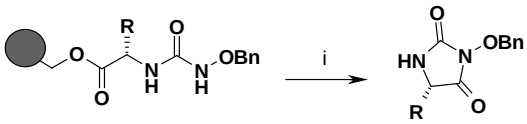
reacting, however, as the experiments performed with piperidine derivatives were also failed, a different reactivity of secondary amines from primary ones was considered as the main reason of the missed success.

Activation of the hydroxylamine with CDI *before* the addition to the linked amine could be a suitable way to accomplish the reactions performed with secondary amines as starting materials.

An interesting behaviour of α-amino acids was found when the release of the title compound was performed by suspending the resin in a solution of $MeOH/NH_3$ (9:1) and stirring overnight at 50°C. The expected methyl ester derivative was never yielded, as shown by the absence of both the ^1H and ^{13}C NMR signals of the methyl ester in the NMR spectrum of the molecule. The acidic O-NH group of the hydroxyurea was probably deprotonated in the reaction environment, and the resulting anion allowed the cyclisation with concomitant release of N-hydroxyhydantoine. (Scheme **8**)

Cyclization never occurred when the cleavage was accomplished using amines as nucleophiles and in case of peptides (as depicted in table **4**); moreover, to confirm the mechanistic hypothesis proposed, experiments will be performed with N-substituted hydroxylamines to avoid the possibility of cyclization.

Intramolecular cyclizations often occur with amino acid ureas giving rise to hydantoine libraries which can be evaluated for their potential as antiviral agents or tumour proliferation inhibitors.[65]



Compound	R
35	$(CH_3)_2CH$
36	$PhCH_2$

Scheme **8**. Reagents and conditions: $MeOH/NH_3$ (9:1), 50°C o/n

[65] a) Chong, P. Y.; Petillo, P. A. *Tet. Lett.* **1999**, *40*, 4501-4504. b) Opačić, N.; Barbarić, M.; Zorc, B.; Cetina, M.; Nagl, A.; Frković, D.; Kralj, M.; Pavelić, K.; Balzarini, J.; Andrei, G.; Snoeck, R.; De Clercq, E.; Raić-Malić, S.; Mintas, M. *J. Med. Chem.* **2005**, *48*, 475-482. c) DeWitt, S. H.; Kiely, J. S.; Stankovic, C. J.; Schroeder, M. C.; Reynolds Cody, D. M.; Pavia, M. R. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6909-6913.

In order to thoroughly explore the intramolecular cyclization, further experiments will be performed starting from β-amino acids with the aim to verify the N-hydroxyhydantoine homologs feasibility.

Finally, to provide a wider method, we started to investigate the reactivity of various resins and hydroxylamines.

Preliminary experiments showed that this procedure is compatible with different solid supports allowing further diversification at the C terminus. Use of acid labile resins, such as Wang and Rink amide, leads to carboxylic acids and to free amides, respectively; moreover, these resins may be applied when a simultaneous removal of all the acid labile protecting groups together with the releasing from the resin is desired.

Thus, either the free or the protected with acid labile groups hydroxylamines were taken into account.

The use of free hydroxylamine avoids the last deprotection step but it has to be further investigated in terms of purification of the final compound.

Although treatment of the activated amino acid with OTBDMS-NH_2 never yielded the desired products, indicating its weak nucleophilic character, the synthesis of TBDMS-protected hydroxyureas may be accomplished by inverting the reaction protocol, consisting in the addition of the TBDMS-protected hydroxylamine pre-activated with CDI to the resin bound free amine.

The synthesis of TBDMS-protected hydroxyureas will be useful to avoid the last hydrogenolytic deprotection and, in case of acid labile solid support, to accomplish the cleavage and the deprotection in only one step. These advantages are of a wide utility to realize automated synthesis and, as a result, to generate in a short time big libraries of organic compounds.

To conclude, since N-hydroxyureas are useful functional groups due to their metal chelating and redox properties, it was developed a new easy and efficient synthetic method on solid-phase to convert Fmoc-protected amino acid derivatives into O-protected N-hydroxyureas through a carbonyldiimidazole activation of the linked amine.

Further experiments will be carried out in order to extend the class of hydroxyureas synthesized and to optimise the procedure. Moreover, it is important to properly define the procedures to yield either the free or the O-TBDMS protected hydroxyureas. Also, diversification of solid phase supports may increase the class of compounds by introducing new elements of molecular diversity.

Once the different aspects of this SPOS procedure will be optimized, we will be able to generate N-hydroxyurea containing molecular libraries important for drug discovery processes.

2.4 Synthesis and screening of bicycle molecular scaffolds (BTAa): an investigation towards new potential inhibitors of Sap2, a major secreted aspartic proteinase from *Candida Albicans*

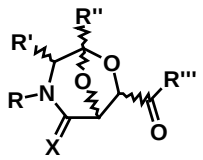
A research project, developed in collaboration with the "Dipartimento di malattie infettive, parassitarie ed immunomediate" of the Istituto Superiore di Sanità in Rome, was set out with the aim to find out new potential Sap2 inhibitors.

The project is based on two different and parallel approaches consisting in a random screening of BTAa libraries and in a rational drug design. As it is a confidential project, the inhibitory activity data of the molecules tested against Sap2 cannot be discuss within the following dissertation.

Random screening of the bicyclic molecular scaffolds against Sap2 activity

One of the two approaches we developed to find out a new potential Sap2 inhibitor consists in a random screening of the bicyclic molecules already present in our chemical library. (Fig. **12**)

The molecules were submitted to spectrophotometric tests performed at Istituto Superiore di Sanità in Rome comparing their inhibitory activity to that of the natural inhibitor Pepstatin A.[66]



X = O; H,H

Figure **12**. General formula of molecules belonging to the BTAa library

[66] Experimental procedure: each assay contained 0.6 ml of 1% (w/v) BSA in 50 mM sodium citrate pH 3.2 and 0.15 ml of enzyme solution (100 mg/ml). After 30 min at 37°C 0.4 ml 10% (w/v) trichloroacetic acid was added. The tubes were stored in ice for 30 min. and then centrifugated (1600 g) for 10 min. The absorbance of the supernatant was read at 280 nm. Negative control: only substrate 1% BSA in citrate buffer. Inhibition control: addition of Pepstatin A (50 mg/ml) to the mixture of enzyme and substrate.

a) Ross, I. K.; De Bernardis, F.; Emerson, G. W.; Cassone, A.; Sullivan, P. A. *J. Gen. Microbiol.* **1990**, *136*, 687-694 ; b) De Bernardis, F.; Agatensi, L.; Ross, I. K.; Emerson, G. W.; Lorenzini, R.; Sullivan, P. A.; Cassone, A. *J. Infect. Dis.* **1990**, *161*, 1276-1283; De Bernardis, F.; Sullivan, P. A.; Cassone, A. *Medical Mycology* **2001**, *39*, 303-313

As the screening of all the compounds would required plenty of time, mixture of molecules belonging to the same family of BTAa were firstly tested in order to highlight the scaffold the most promising as Sap2 inhibitor.

Since we had first evidence that a precise class of scaffolds showed a fair inhibitory activity, the screening of single compounds was set out to identify the best flanking units for scaffold decoration and to get more insights into the structure-activity relationship. Thanks to the screening results we were able to highlight some hit compounds and, consequently, we planned some modifications on these hits in order to better define their structural features in terms of binding. Small molecular libraries were so synthesized with the aim to develop the procedure from hit to lead and to have further insight into the structure-activity relationship.

Rational drug design

Sap2 displays sensitivity towards the natural inhibitor Pepstatin A, an extended hexapeptide which acts as a tetrahedral transition state mimic by virtue of a central statine residue containing the non scissile bond CHOH-CH_2. The hydroxyl group forms hydrogen bonds with the two catalytic aspartate side chains. Pepstatin is a non specific inhibitor of aspartic proteases and because of its non-selectivity and lack of potency and safety it is not an ideal agent for therapy.

Abbott laboratories developed one of the most potent inhibitor of Sap2, A-70450 which is a pseudo hexapeptide in which the peptide bond to be hydrolysed is replaced by the hydroxyethylene isostere.

Other noteworthy features of A-70450 include a lactam ring that severely restricts the conformation around the P3 residue, the benzyl side chain of P3 having the *R* configuration, a piperazine substituent at P4, and a *n*-butyl side chain at P2. (Fig. **13**, **A**)

The three dimensional structure of a close homologue of Sap2 from *C. Albicans* complexed either with the potent inhibitor A-70450 or with Pepstatin A, provided considerable insight into the interactions of the ligand with the active site of the enzyme.[67]

[67] a) Cutfield, S. M.; Dodson, E. J.; Anderson, B. F.; Moody, P. C. E.; Marshall, C. J.; Sullivan, P. A.; Cutfield, J. F. *Structure*, **1995**, *3*, 1261-1271; b) Pranav Kumar, S. K.; Kulkarni, V. M. *Bioorg. Med. Chem.* **2002**, *10*, 1153-1170; c) Abad-Zapatero, C.; Goldman, R.; Muchmore, S. W.; Hutchins, C.; Stewart, K.; Navaza, J.; Payne, C. D.; Ray, T. L. *Protein Science* **1996**, 5, 640-652

On the base of these studies we planned the synthesis of new peptidomimetics as bioisosteres of the tight-binding inhibitor A-70450. These peptidomimetics were designed by inserting the bicycle lactam on the ketopiperazine of the target molecule. (Figure **13**, **B**)

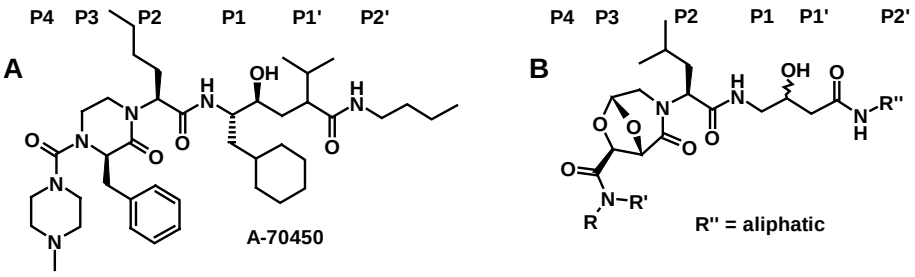
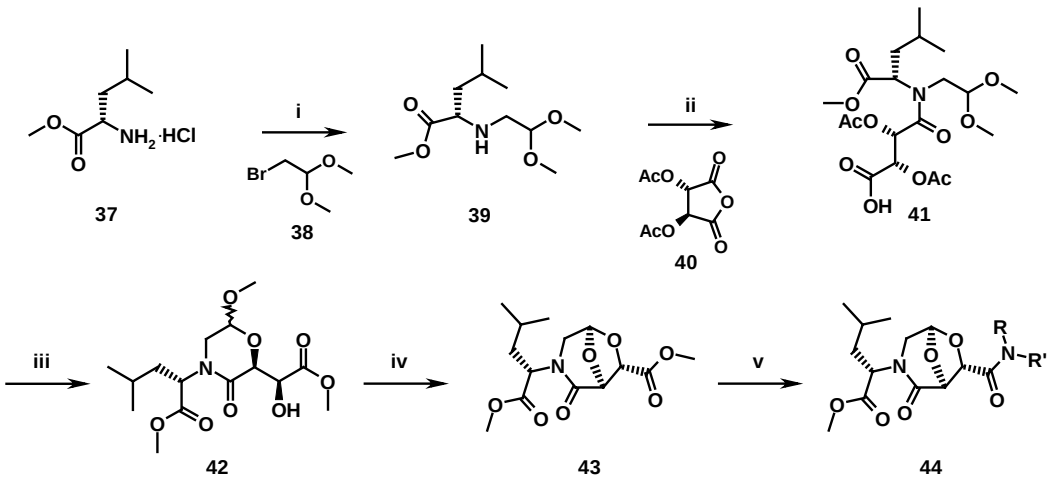


Figure **13**. The tight-binding inhibitor A-70450 (A) and the general formula of its potential bioisosteres based on the BTAa scaffold (B)

The synthesis of the bicycle molecular scaffold **43** was accomplished following the same procedure described for compound **18**. (Scheme **8**) Thanks to the high reactivity of the methyl ester in 7,[68] synthesis of amides (**44**) was accomplished without using activating agents with an efficient and selective procedure. Several amides were so inserted in 7 with the aim to explore the S4 binding pocket.

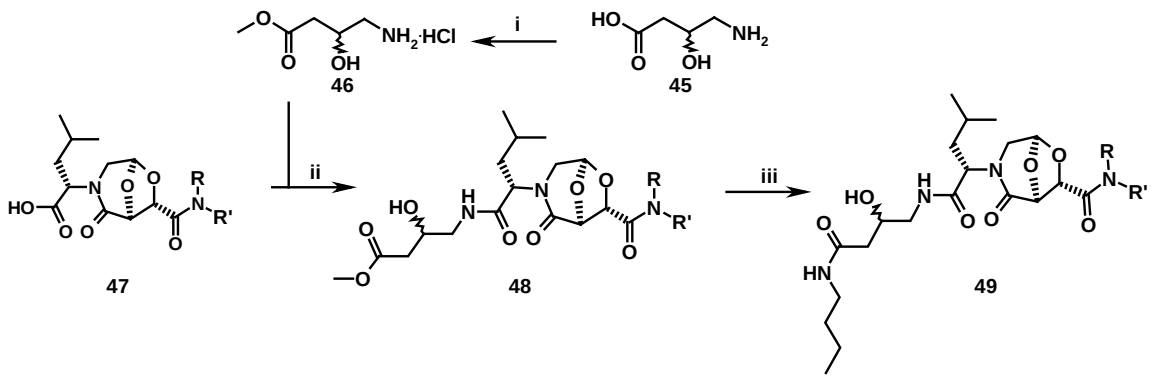


Scheme **8**. Reagents and conditions: (i) NEt_3, KI cat., DMF, 120°C (28%); (ii) DCM, r.t. o/n; (iii) $SOCl_2$, MeOH, 60°C, 1 h; (iv) SiO_2/H^+, toluene, reflux, 20 min (50% over three steps); N-RR', 40°C, o/n

[68] Machetti, F.; Bucelli, I.; Indiani, G.; Guarna, A. *Chimie* **2003**, *6*, 631-633

Once highlighted the best P4 residue to insert in position 7 of the scaffold, decoration of the opposite part of the bicycle ring with a statine derivative was accomplished.

A coupling reaction between the carboxylic acid **47** and the (3*R*/*S*)-4-amino-3-hydroxy-butyric acid methyl ester **46** afforded the statine derivative **48** as a mixture of two diastereoisomers. Treatment of **48** with *n*-butyl amine at 50°C in a mixture of THF and a catalytic amount of water yielded the title compound **49** in good yield. (Scheme **9**)



Scheme **9**. Reagents and conditions: (i) $SOCl_2$, MeOH, r.t., o/n; (ii) PyBROP, DIPEA, DCM, r.t., o/n (44%); (iii) *n*$BuNH_2$, THF/H_2O, 50°C, o/n (74%)

Comparison of the inhibition of Sap2 activity on behalf of the mixture of the two diastereisomers **49** to that of Pepstatin A gave a first evidence of a quite good ligand-enzyme interaction.

Chapter 3

CONCLUSIONS

New compounds with affinity for the catalytic domain of the MMP-12 were designed using an integrated approach based on virtual screening and on NMR analysis. The molecules are built around a BTAa scaffold decorated with a biphenylic moiety and with a ZBG. The adducts provided by docking calculation enabled the identification of the catalytic pocket of MMP-12 as the binding site for this class of compounds. The inspection of the residues showing a significant chemical shift perturbation supports this picture, with the lipophilic moiety fitted into S1' cavity and the bicyclic skeleton located outside, in front of the catalytic zinc.

Among the synthesized compounds, the scaffold **5** having the N-hydroxyurea group in position 3 and the *p*-phenylbenzylcarboxy amide in position 7 showed an improved affinity (K_D = 154 μM and IC_{50} 149 μM) for MMP-12 and some selectivity towards the other MMPs. The X-ray structure of the catalytic domain of Human Macrophage Metalloelastase in the presence of hydroxyurea derivative **5** gave more insight into the ligand-enzyme interaction and appeared as a new starting point for further MMP-12 inhibitors design.

The insertion of a methylene group between the bicycle ring and the ZBG (compound **6**) provided a weak improvement in the activity even if the main binding contribution was still determined by the insertion of the biphenylic moiety in the S1' cavity.

Further experiments will be accomplished to better anchor the zinc chelating group in order to find its best spatial orientation for a strong electrostatic interaction with the catalytic ion. In this respect, the other BTAa isomers will be properly decorated and new organic linkers will be introduced between the scaffold and the ZBG.

Moreover, a molecular library of BTAa based compounds bearing several different lipophilic groups at position 7 will be easily generate in order to optimise the fitting in the S1' pocket.

Also, other fragments may be easily inserted in further positions of the bycicle skeleton to better interact with the enzyme backbone and to selectively accommodate into the binding pocket of MMP-12.

The synthetic versatility of BTAas, taken together with dissociation constant in a micromolar range and fair selectivity, candidate these molecules as new compound guides to develop more potent and selective inhibitors for MMP-12.

As conformationally constrained hydroxamic acids related to proline scaffold are interesting compounds for MMP inhibition, the synthesis of new proline mimetics based on the bicycle molecular scaffold was set out starting from an L-ascorbic acid derivative.

The introduction of proper pharmacophore groups on the new α-amino acid molecular scaffolds may represent an alternative route for the research of new potential selective MMP-12 inhibitors.
Moreover, these new BTAa based scaffolds may find application in peptidomimetic research, and, in particular, are suited for solid-phase organic and peptide synthesis by the Fmoc protocol.

Since N-hydroxyureas are useful functional groups thanks to their metal chelating and redox properties, it was developed a new easy and efficient Solid Phase Organic Synthesis method to convert Fmoc-protected amino acids to O-protected N-hydroxyureas through the carbonyldiimidazole activation of supported amines.
Further experiments will be carried out in order to optimise different aspects of this solid phase procedure and with the aim to extend the class of N-hydroxyureas which may be synthesized.
In this respect, it will be properly defined the procedure to yield either the free or the O-TBDMS protected hydroxyureas. Indeed, the use of the free or the O-TBDMS protected hydroxylamine is of major utility as it allows to avoid the hydrogenolytic cleavage of the benzyl protecting group and, consequently, leads to easier procedures which may be applied in automated systems for the generation of combinatorial libraries.
Also, diversification of solid phase supports may increase the class of compounds which can be synthesized and so enhance the degree of molecular diversification.
Once the different aspects of this SPOS procedure will be optimized, we will be able to generate N-hydroxyurea containing molecular libraries important for drug discovery processes.

The random screening of BTAa derivatives, followed by a more focussed screening of scaffolds with specific structural features, allowed to complete a thorough collection of data about Sap2 inhibition and to gain a preliminary and deep knowledge concerning the structure activity relationship.
On parallel, according to a rational drug design, a bioisostere of the tight binding inhibitor A-70450 was synthesized and its fair inhibitory activity suggested that the insertion of the bicycle scaffold on the ketopiperazine ring of the target molecule may actually be an interesting route for new Sap2 inhibitors research.

Further investigations will be done to optimise the bioisostere structure improving its binding with the catalytic site of the enzyme.

First of all, the two diastereoisomers which were synthesized as a mixture of compounds will be separated and then tested again. It is well known, in fact, that the stereochemistry of the central statine residue is extremely important for a good binding.

Moreover, a small peptidomimetic library varying the P2' residue (see Fig. 13, B) will be generate with the aim to improve specificity.It is reported, in fact, that the fitting of the P2' grouop into the corresponding pocket may contribute in the selectivity towards the other aspartyl proteases, such as Cathepsin D and Renin.

Either the random screening or the rational drug design approach suggested that BTAa, thanks to their synthetic versatility, may represent a new type of scaffold to design new aspartyl protease inhibitors.

EXPERIMENTAL

Melting points are uncorrected and were measured on microscope RCH Kofler apparatus. Chromatographic purifications were performed with silica gel 60 (0.040-0.063 mm), unless otherwise stated, using flash column techniques; all TLC development was performed on silica gel coated (Merck Silica gel 60 F_{254}, 0.25 mm) sheets. IR spectra were recorded on Perkin Elmer 881 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Gemini 200 (200 MHz for ^{1}H and 50.33 MHz for ^{13}C) instrument or a Varian Mercury 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) instrument using tetramethylsilane as internal standard and the chemical shifts were reported in δ (ppm) units. EI Mass spectra were carried out at 70 eV ionizing voltage by direct introduction on a QMD 1000 Carlo Erba instrument, whereas ESI-MS spectra were carried out on LTQ Thermo Finnigan instrument. Microanalysis were carried out on Perkin Elmer 240C elemental analyser. Optical rotation measurements were performed on JASCO DIP-370 digital polarimeter. All reactions requiring anhydrous conditions were carried out under N_2 atmosphere and were performed in oven-dried glassware.

HPLC analysis were performed with a Beckman-Gold system, mobile phase: H_2O/TFA 0.001% and CH_3CN/TFA 0.001%; Jasco U.V. detector, λ = 230 nm; Alltech Econosphere riverse phase C18 5U column (250 mm × 4.6 mm), 20 μL loader. The program used was: CH_3CN/TFA 10%-20 min-90%-5 min-90%-5 min-10%, rate flow 1 mL/min.

Preparation of acid silica gel (SiO_2/H_2SO_4): SiO_2 (29 g) was suspended in CH_2Cl_2 (400 ml) with 12 g of H_2SO_4 and left under quickly magnetic stirring for 40 min. The suspension was then concentrated at low pressure.

(2,2-Dimethoxy-ethylamino)-acetic acid methyl ester (14)

A suspension containing glycine methyl ester hydrochloride (4 g, 31.8 mmol), NEt_3 (4.43 mL, 31.8 mmol), dimethoxy acetaldehyde (2.85 mL, 31.5 mmol) and Pd/C 10% in 100 mL of MeOH was stirred at room temperature overnight under H_2 atmosphere. Filtration through a pad of celite afforded the crude product. Further purification by column chromatography (Silica gel, AcOEt/EP 4:1) yielded the pure **14** as a colorless oil (3.2 g, 59% yield).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 4.41 (t, J = 5.3 Hz, 1H), 3.68 (s, 3H), 3.40 (s, 2H), 3.33 (s, 6H), 2.71 (d, J = 5.6 Hz, 2H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ = 172.5 (s), 103.8 (d), 53.84 (q), 51.82 (q), 50.75 (t), 50.62 (t); IR ($CHCl_3$) 3336, 2834, 1737, 1356 MS *m/z* 118 (5.98), 102 (4.49), 75 (100).

***R*-hydroxy-[(2*R*, 6*R*/*S*)-(6-methoxy-4-methoxycarbonylmethyl-3-oxo-morpholin-2-yl)]-acetic acid methyl ester (17)**

To a suspension of (*R*,*R*)-2,3-di-O-acetyltartaric anhydride (**15**) (3.9 g, 18 mmol) in dry DCM (18 ml) was added, at 0 °C and under N_2 atmosphere, a solution of **14** (3.2 g, 18 mmol) in dry DCM (9 ml). The reaction mixture was stirred at room temperature overnight. After evaporation of the solvent, the crude product was dissolved in MeOH (30 ml) and thionyl chloride (1.12 mL, 15 mmol) was added drop wise at 0 °C. The mixture was then allowed to reach 60 °C and stirred for 2 h. The solvent was removed and the crude product was isolated as a yellow oil and used without further purification in the next step. Purification of the crude product by column chromatography (Silica gel, AcOEt/EP 4:1) afforded the pure **17** as an oil.

$[\alpha]_D^{26}$ + 121.03 (*c* 1.1, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 4.89 (d, J = 3 Hz, 1H), 4.82 (s, 1H), 4.54 (s, 1H), 4.25 (d, J = 17.2 Hz, 1H), 3.96 (d, J = 17.2 Hz, 1H), 3.77 (s, 3H), 3.68 (s, 3H), 3.46-3.39 (m, 1H), 3.43 (s, 3H), 3.21 (d, J = 12.2 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ = 172.1 (s), 168.5 (s), 165.9 (s), 94.78 (d), 72.20 (d), 71.32 (d), 55.13 (q), 52.75 (q), 52.35 (q), 51.26 (t), 47.86 (t); IR ($CHCl_3$) 3545, 1745, 1665, 1266, 1141 cm^{-1}; MS *m/z* 291 (M, 0.63), 260 (11.55), 232 (26.05), 58 (100); Anal. Calcd for $C_{11}H_{17}NO_8$ (291.26): C, 45.36; H, 5.88; N, 4.81. Found: C, 44.03; H, 6.03; N, 4.44.

(1*R*, 5*S*, 7*R*)-3-methoxycarbonylmethyl-2-oxo-6,8-dioxa-3-aza-bicyclo[3.2.1]octane-7-carboxylic acid methyl ester (18)

A solution of **17** (5.2 g, 18 mmol) in toluene (25 ml) was quickly added to a refluxing suspension of SiO_2/H_2SO_4 (4 g) in toluene (50 ml). The mixture was allowed to react for 30' and then one-third of the solvent was distilled off. The hot reaction mixture was filtered through a pad of $NaHCO_3$ and, after evaporation of the solvent, the crude product was purified by flash chromatography (Silica gel, AcOEt/EP 2:1) affording **18** as a colorless oil (800 mg, 17% yield over three steps).

$[\alpha]_D^{24}$ -49.9 (*c* 1.13, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 5.83 (d, J = 2.2 Hz, 1H), 4.83 (s, 1H), 4.68 (s, 1H), 4.08-3.86 (AB system, 2H), 3.69 (s, 3H), 3.64 (s, 3H), 3.52 (dd, J_1 = 11.6 Hz, J_2 = 2.2 Hz, 1H), 3.21 (d, J_1 = 11.6 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ = 171.6 (s), 168.9 (s), 168.2 (s), 165.9 (s), 99.88 (d), 77.59 (d), 72.23 (d), 52.90 (q), 52.81 (t), 52.40 (q), 46.16 (t); IR ($CHCl_3$) 1749, 1683, 1136, 1108 MS *m/z* 259 (M, 5.39), 200

(13.01), 102 (100); Anal. Calcd for $C_{10}H_{13}NO_7$ (259.22)**:** C, 46.34; H, 5.06; N, 5.40. Found: C, 45.60; H, 5.37; N, 5.44.

(1*R*, 5*S*, 7*R*)-7-[(Biphenyl-4-ylmethyl)-carbamoyl]-2-oxo-6,8-dioxa-3-aza-bicyclo[3.2.1]oct-3-yl}-acetic acid methyl ester (19)

A solution containing **18** (80 mg, 0.3 mmol) and p-phenylbenzyl amine (68 mg, 0.37 mmol) in DCM (200 µl) was stirred at 40°C overnight. After evaporation of the solvent, the crude product was purified by flash chromatography (Silica gel, DCM/MeOH 20:1) affording **19** as a white solid (108 mg, 88% yield).

Mp 193-196 °C; $[\alpha]_D^{24}$ +13.8 (*c* 0.98, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 7.60-7.31 (m, 9H), 6.86 (bs, 1H), 5.87 (d, J = 2.2 Hz, 1H), 5.07 (s, 1H), 4.76 (s, 1H), 4.52 (d, J 5.8 Hz, 2H), 4.20 (d, J = 17.6 Hz, 1H), 3.94 (d, J = 17.6 Hz, 1H), 3.75 (s, 3H), 3.60 (dd, J_1 = 11.8 Hz, J_2 = 1.8 Hz, 1H), 3.31 (d, J_1 = 11.8 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ = 168.1 (s), 167.9 (s), 165.9 (s), 140.3 (s), 136.2 (s), 128.5, 127.8, 127.2, 127.1, 126.7 (d), 99.52 (d), 79.08 (d), 77.08 (d), 52.39 (t), 52.15 (q), 45.70 (t), 42.74 (t); IR ($CHCl_3$) 1748, 1686, 1520 MS *m/*z 410 (M, 19.24), 182 (71.98), 167 (100); Anal. Calcd for $C_{22}H_{22}N_2O_6$ (410.43): C, 64.38; H, 5.40; N, 6.83. Found: C, 64.21; H, 5.86; N, 5.46.

(1*R*,5*S*,7*R*)-3-{2-[(benzyloxy)amino]-2-oxoethyl}-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]octane-7-carboxamide (20)

To a solution of **19** (220 mg, 0.54 mmol) in MeOH/THF (2:3) was added LiOH (96 mg, 2.3 mmol) and the resulting mixture was stirred at room temperature overnight.

The reaction mixture was then diluted with aqueous 5% HCl and the organic solvent was evaporated under reduced pressure; the aqueous layer was then washed with $CHCl_3$ to extract the product. The resulting organic layer was dried over Na_2SO_4 and, after evaporation of the solvent, 100 mg (0.25 mmol) of the crude ((1*R*,5*S*,7R)-7-{[(1,1'-biphenyl-4-ylmethyl)amino]carbonyl}-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]oct-3-yl)acetic acid were dissolved at 0°C in a solution containing HOBT (41 mg, 0.5 mmol), DIC (77 µL, 0.5 mmol), benzylhydroxylamine (31 mg, 0.25 mmol) and DIPEA (150 µL, 0.88 mmol) in DCM (2 mL). The resulting solution was allowed to reach room temperature and was stirred overnight. The reaction mixture was then washed with a saturated solution of $NaHCO_3$, aqueous 5% $KHSO_4$ and brine and dried over Na_2SO_4. After evaporation of the solvent the crude product was purified by flash chromatography (silica gel, AcOEt/EP 3:1)

to afford **20** (50 mg, 42% yield in two steps) which was directly used in the next step without a complete characterization.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.61-7.50 (m, 5H), 7.48-7.24 (m, 9H), 7.00-6.88 (m, 1H), 5.86 (s, 1H), 5.04 (s, 1H), 4.78 (s, 1H), 4.58-4.44 (m, 2H), 3.90-3.73 (m, 2H), 3.61-3.46 (m, 1H), 3.33-3.12 (m, 1H); MS *m/z* 410 (6.15), 379 (1.73), 339 (1.44), 167 (100).

(1*R*,5*S*,7*R*)-3-Hydroxycarbamoylmethyl-2-oxo-6,8-dioxa-3-aza-bicyclo[3.2.1]octane-7-carboxylic acid (biphenyl-4-ylmethyl)-amide (6)

A stirred solution of **20** (50 mg, 0.1 mmol) in THF (3 mL) was submitted to hydrogenolysis over 10% Pd/C at 1Atm, at room temperature, over night. After separation of the catalyst by filtration through a pad of celite, the solvent was evaporated affording **6** as a white solid (25 mg, 61% yield).

^{1}H-NMR (DMSO, 200 MHz): δ 8.77-8.61 (m, 1H), 7.65-7.43 (m, 4H), 7.40-7.27 (m, 5H), 6.03 (bs, 1H), 4.67 (s, 1H), 4.60 (s, 1H), 4.32 (d, J = 5.4 Hz, 2H), 3.39-3.42 (m, 2H), 3.22-3.13 (m, 2H); MS *m/z* 410 (3.31), 395 (0.65), 338 (28.8), 167 (100).

(2S)-2-({[(benzyloxy)amino]carbonyl}amino)-N-ethyl-3-phenylpropanamide (31)

HMBA-AM resin **25** (200 mg, 0.17 mmol) was preswelled in 3 mL of DMF. After draining off the solvent, a solution of Fmoc-L-PheOH (263 mg, 0.68 mmol), HOBT (92 mg, 0.68 mmol), DIC (107 μL, 0.68 mmol) and a catalytic amount of DMAP in 3 mL of DMF was added and the resulting suspension was stirred overnight at room temperature. The solution was then filtered off and the derivatized resin was washed with DMF. Next, the resin was shaken for 30 min. at r.t. with 20% piperidine in DMF to carry out Fmoc deprotection and after draining off the solution it was thoroughly washed with DMF, MeOH and DCM. Crude resin was then treated at 0°C with a solution of CDI (83 mg, 0.51 mmol) in 3 mL of DCM and the resulting mixture was allowed to reach room temperature and stirred for 1 hour. After removing of the solution and washing with DCM and THF, $BnONH_2$ (63 mg, 0.51 mmol), as a free base, in 2 mL of DCM was added to the resin and the mixture was shaken overnight at r.t. Then the solution was filtered off and the resin was washed with DCM and THF. Releasing of the title compound was performed by suspending the resin in a solution of $EtNH_2$ (70% in H_2O, 1.5 mL) in THF and stirring at room temperature overnight. After filtering out the solution and eluting the resin with THF, the combined organic layers were evaporated under reduced pressure affording a pale

yellow oil. Chromatographic purification (AcOEt/EP, 2:1) afforded pure **31** as a white solid (23 mg, 40% yield).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.37-7.18 (m, 10H), 7.08 (s, 1H), 6.14 (d, J = 8 Hz, 1H), 5.55 (bs, 1H), 4.76-4.64 (AB system, 2H), 4.42 (pseudo q, J = 7.3 Hz, 1H), 3.22-3.09 (m, 3H), 2.94 (dd, J_1 = 13.6 Hz, J_2 = 7.8 Hz, 1H), 0.97 (t, J = 7.4 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 170.2 (s), 159.1 (s), 136.5 (s), 135.0 (s), 129.3, 128.8, 128.6, 127.0 (d), 78.68 (t), 54.75 (d), 38.61 (t), 34.48 (t), 14.72 (q); MS *m/z* 341 (7.46), 91 (100).

(2*S*)-2-({[(benzyloxy)amino]carbonyl}amino)-N-isobutyl-2-phenylethanamide (32)

Compound **32** was prepared according to the procedure described for **31** starting from HMBA-AM resin **25** (100 mg, 0.08 mmol) and Fmoc-L-PheGly-OH (119 mg, 0.32 mmol). Releasing of the title compound was performed by suspending the resin in a solution of isobutyl amine (165 μL, 1.66 mmol) in 2 mL of THF and a catalytic amount of water and stirring at room temperature overnight. After filtering out the solution and eluting the resin with THF, the combined organic layers were evaporated under reduced pressure affording a pale yellow oil. The purity obtained after cleavage was 50%, as indicated by HPLC analysis. Further chromatographic purification (AcOEt/EP, 1:1) afforded pure **32** as a colorless oil (8 mg, 28% yield).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.42-7.34 (m, 5H), 7.33-7.22 (m, 5H), 7.01 (d, J = 4 Hz, 1H), 6.96 (s, 1H), 5.88 (bs, 1H), 5.30 (d, J = 7 Hz, 1H), 4.89-4.77 (AB system, 2H), 3.16-2.92 (m, 2H), 1.75-1.58 (m, 1H), 0.79 (d, J = 6.6 Hz, 6H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 169.6 (s), 158.9 (s), 138.2 (s), 135.2 (s), 129.4, 128.9, 128.8, 128.7, 128.2, 127.0 (d), 78.77 (t), 57.51 (d), 47.20 (t), 28.56 (d), 20.08 (q); MS *m/z* 355(3.70), 255 (24.15), 233 (7.74), 149(56.11), 91 (100).

({[(benzyloxy)amino]carbonyl}amino)-Phe-Val-OMe (33)

HMBA-AM resin **25** (200 mg, 0.17 mmol) was preswelled in 3 mL of DMF. After draining off the solvent, a solution of Fmoc-L-Val-OH (231 mg, 0.68 mmol), HOBT (92 mg, 0.68 mmol), DIC (107 μL, 0.68 mmol) and a catalytic amount of DMAP in 3 mL of DMF was added and the resulting suspension was stirred overnight at room temperature. The solution was then filtered off and the derivatized resin was washed with DMF. Next, the resin was shaken for 30 min. at r.t. with 20% piperidine in DMF to carry out Fmoc deprotection. After draining off the solution and washing the resin with DMF, Fmoc-L-Phe-OH (263 mg, 0.68 mmol) was loaded onto the resin using the same coupling

conditions described above. After Fmoc deprotection, the resin was filtered off and thoroughly washed with DMF, MeOH and DCM. Crude resin was then treated at 0°C with a solution of CDI (83 mg, 0.51 mmol) in 3 mL of DCM and the resulting mixture was allowed to reach room temperature and stirred for 1 hour. After removal of the solution and washing with DCM and THF, $BnONH_2$ (63 mg, 0.51 mmol), as a free base, in 2 mL of DCM was added to the resin and the mixture was shaken overnight at r.t. Then the solution was filtered off and the resin was washed with DCM and THF. Releasing of the title compound was performed by suspending the resin in a solution of $MeOH/NH_3$ (9:1, 10 mL) and stirring overnight at 50°C. After filtering out the solution and eluting the resin with MeOH, the combined organic layers were evaporated under reduced pressure affording a pale yellow oil. Chromatographic purification (AcOEt/EP, 1:1) afforded pure **33** (60 mg, 83% yield) as a colorless oil.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.40-7.17 (m, 11H), 6.63 (d, J = 8.4 Hz, 1H), 6.22 (d, J = 7.6 Hz, 1H), 4.69 (s, 2H), 4.60 (pseudo q, J = 7.3 Hz, 1H), 4.43 (dd, J_1 = 8.6 Hz, J_2 = 5.6 Hz, 1H), 3.68 (s, 3H), 3.04 (d, J = 7 Hz, 2H), 2.17-2.04 (m, 1H), 0.84 (t, J = 6.8 Hz, 6H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 171.6 (s), 170.7 (s), 159.2 (s), 136.4 (s), 135.0 (s), 129.3, 129.1, 128.8, 128.6, 128.6, 126.9 (d), 78.73 (t), 57.47 (d), 54.65 (d), 52.17 (q), 38.27 (t), 31.29 (d), 19.00 (q), 17.98 (q); MS *m/z* 427 (6.42), 297 (5.93), 91 (100).

Methyl 4-[({[(benzyloxy)amino]carbonyl}amino)methyl]benzoate (34)

Compound **34** was prepared according to the procedure described for **31** starting from HMBA-AM resin **25** (100 mg, 0.08 mmol) and 4-((fmoc-amino)methyl)benzoic acid (119 mg, 0.32 mmol). Releasing of the title compound was performed by suspending the resin in a solution of $MeOH/NH_3$ (9:1, 10 mL) and stirring overnight at 50°C. After filtering out the solution and eluting the resin with MeOH, the combined organic layers were evaporated under reduced pressure affording a pale yellow oil. The purity obtained after cleavage was 51%, as indicated by HPLC analysis. Further chromatographic purification (AcOEt/EP, 1:1) afforded pure **34** (11 mg, 44%).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.97 (d, J = 8.2 Hz, 2H), 7.36 (s, 5H), 7.20 (d, J = 8.2 Hz, 2H), 7.06 (s, 1H), 5.93 (bs, 1H), 4.80 (s, 2H), 4.42 (d, J = 6.2 Hz, 2H), 3.91 (s, 3H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 159.4 (s), 143.2 (s), 134.8 (s), 129.6, 129.0, 128.7, 128.5, 126.8 (d), 78.48 (t), 51.86 (q), 42.88 (t) MS *m/z* 314 (0.14), 283 (2.35), 164 (4.58), 91 (100).

(5*S*)- 3-Benzyloxy-5-isopropyl-imidazolidine-2,4-dione (35)

Compound **35** was prepared according to the procedure described for **31** starting from HMBA-AM resin **25** (200 mg, 0.17 mmol) and Fmoc-L-ValOH (231 mg, 0.68 mmol).

Releasing of the title compound was performed by suspending the resin in a solution of $MeOH/NH_3$ (9:1, 10 mL) and stirring overnight at 50°C. After filtering out the solution and eluting the resin with MeOH, the combined organic layers were evaporated under reduced pressure affording a pale yellow oil. Chromatographic purification (AcOEt/EP, 1:1) afforded pure **35** as a white solid (19 mg, 45%).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.51-7.43 (m, 2H), 7.40-7.31 (m, 3H), 5.71 (s, 1H), 5.13 (s, 2H), 3.82 (d, J = 3.8 Hz, 1H), 2.24-2.09 (m, 1H), 0.98 (d, J = 6.8 Hz, 3H), 0.83 (d, J = 7.0 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 167.5 (s), 154.3 (s), 133.3 (s), 129.8, 129.3, 128.4 (d), 79.31 (t), 60.44 (d), 30.24 (d), 18.51 (q), 16.10 (q); MS *m/z* 141 (0.80), 100 (10.39), 91 (100).

(5*S*)- 5-Benzyl-3-benzyloxy-imidazolidine-2,4-dione (36)

Compound **36** was prepared according to the procedure described for **31** starting from HMBA-AM resin **25** (200 mg, 0.17 mmol) and Fmoc-L-PheOH (263 mg, 0.68 mmol).

Releasing of the title compound was performed by suspending the resin in a solution of $MeOH/NH_3$ (9:1, 10 mL) and stirring overnight at 50°C. After filtering out the solution and eluting the resin with MeOH, the combined organic layers were evaporated under reduced pressure affording a pale yellow oil. Filtration over a pad of Amberlyst 15 afforded 45 mg of **36** as a pale yellow oil.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.46-7.10 (m, 10H), 6.02 (s, 1H), 4.98-4.87 (AB system, 2H), 4.14 (dd, J_1 = 8.2 Hz, J_2 = 4 Hz, 1H), 3.18 (dd, J_1 = 13.8 Hz, J_2 = 4 Hz, 1H), 2.79 (dd, J_1 = 14 Hz, J_2 = 8.2 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 167.2 (s), 153.2 (s), 134.4 (s), 133.2 (s), 129.9, 129.4, 129.2, 128.8, 128.5, 128.4 (d), 79.35 (t), 56.24 (d), 37.71 (t).

(2 *S*)-2-(2',2'-dimethoxy-ethylamino)-4-methyl-pentanoic acid methyl ester (39)

A solution containing L-leucine methyl ester hydrochloride (2.9 g, 16 mmol), 2-bromo-1,1-dimethoxy ethane (1.9 ml, 2.7 g, 16 mmol), NEt_3 (6.7 ml, 48 mmol) and a catalytic amount of KI in DMF (190 mL) was stirred at 120 °C for 3 days. The reaction mixture was concentrated under reduced pressure, diluted with water and extracted with DCM. The organic layer was then washed with brine, dried over Na_2SO_4 and evaporated. The crude

product was purified by column chromatography (silica gel, AcOEt/EP 1:1) to afford **39** as a yellow oil (1.2 g, 32% yield).

$[\alpha]_D^{24}$ -3.32 (*c* 1.02, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 4.38 (t, J = 6 Hz, 1H), 3.65 (s, 3H), 3.30 (s, 3H), 3.29 (s, 3H), 3.24 (t, J = 6 Hz, 1H), 2.68 (dd, $J_1 = J_2 = 6$ Hz, 1H), 2.52 (dd, $J_1 = J_2 = 6$ Hz, 1H), 1.71-1.55 (m, 2H), 1.44-1.37 (m, 2H), 0.86 (d, J = 4 Hz, 3H), 0.83 (d, J = 4 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 175.9 (s), 103.6 (d), 59.94 (d), 54.02 (q), 53.08 (q), 51.67 (q), 49.26 (t), 42.78 (t), 24.99 (d), 22.76 (q), 22.48 (q); MS *m/z* 233 (0.46), 202 (7.16), 174 (33.8), 158 (13.9), 75 (100); IR ($CHCl_3$) 3000-2831, 1729, 1464-1435, 1130, 1065 cm^{-1}; Anal. Calcd for $C_{11}H_{23}NO_4$ (233.30): C, 56.63; H, 9.94; N, 6.00. Found: C, 57.49; H, 9.90; N, 6.24.

(2*S*)-2-[(2'*S*)-2'-(hydroxy-methoxycarbonyl-methyl)-6-methoxy-3-oxo-morpholin-4-yl]-4-methyl pentanoic acid methyl ester (42)

To a suspension of (*S*,*S*)-2,3-di-O-acetyltartaric anhydride (**40**) (1 g, 4.7 mmol) in dry DCM (4.5 ml) was added, at 0 °C and under N_2 atmosphere, a solution of **39** (1 g, 4.7 mmol) in dry DCM (2.5 ml). The reaction mixture was stirred at room temperature overnight. After evaporation of the solvent, the crude product was dissolved in MeOH (8 ml) and thionyl chloride (292 μL, 4 mmol) was added drop wise at 0 °C. The mixture was then allowed to reach 60 °C and stirred for 2 h. The solvent was removed and the crude **42** was isolated as a yellow oil and used without further purification in the next step.

(1*R*, 5*S*, 7*S*)-3-[(1'*S*)-1'-Methoxycarbonyl-3'-methyl-butyl]-2-oxo-6,8-dioxa-3-aza-bicyclo[3.2.1]octane-7-carboxylic acid methyl ester (43)

A solution of **42** (1.63 g, 4.7 mmol) in toluene (8 ml) was quickly added to a refluxing suspension of SiO_2/H_2SO_4 (1 g) in toluene (12 ml). The mixture was allowed to react for 30' and then the one-third of the solvent was distilled off. The hot reaction mixture was filtered through a pad of $NaHCO_3$ and, after evaporation of the solvent, the crude product was purified by flash chromatography (Silica gel, AcOEt/EP 1:2) affording **43** as a white solid (730 mg, 50% yield over three steps).

$[\alpha]_D^{24}$ 22.0 (*c* 1.01, MeOH); ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 5.88 (d, J = 2 Hz, 1H), 5.09 (t, J = 8 Hz, 1H), 4.87 (s, 1H), 4.59 (s, 1H), 3.72 (s, 3H), 3.64 (s, 3H), 3.50 (dd, $J_1 = 12$ Hz, $J_2 = 2$ Hz, 1H), 3.11 (dd, $J_1 = 12$ Hz, 1H), 1.67-1.60 (m, 2H), 1.46-1.32 (m, 1H), 0.88 (s, 3H), 0.84 (s, 3H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 170.8 (s), 168.7 (s), 165.6 (s), 100.0 (d),

77.77 (d), 77.35 (d), 52.82 (d), 52.43 (q), 52.26 (q), 48.06 (t), 36.62 (t), 24.75 (d), 23.29 (q), 21.28 (q); MS *m/z* 315 (11.0), 256 (100), 240 (3.94); Anal. Calcd for $C_{14}H_{21}NO_7$ (315.33): C, 53.33; H, 6.71; N, 4.44. Found: C, 52.99; H, 5.58; N, 4.79.

General procedure for amides (44) synthesis

A solution containing **43** (1 g, 3.2 mmol) and the desired amine (63 mmol) was stirred at 60°C over night. The reaction mixture was then concentrated under reduced pressure and the crude product was purified by column chromatography (silica gel, DCM/ MeOH 20:1) to afford derivatives **44** in good yield.

In the following NMR characterization the signals corresponding to R and R' groups are omitted. ^{1}H-NMR ($CDCl_3$, 200 MHz): (mixture of two rotamers) δ 5.79 (d, 1H, J = 1.4 Hz), 5.06-4.94 (m, 1H), 5.02 (s, 1H), 4.82 (s, 1H, minor rotamer), 4.71 (s, 1H, major rotamer), 3.62 (s, 3H, minor r.), 3.61 (s, 3H, major r.), 3.55-3.20 (m, 1H), 3.09 (d, J = 11.8 Hz, 1H), 1.67-1.34 (m, 3H), 0.86 (d, J = 4.8 Hz, 3H), 0.84 (d, J = 5.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 200 MHz) (mixture of two rotamers): δ 171.1 (s, minor r.), 170.8 (s, major r.), 167.6 (s, minor r.), 166.8 (s, major r., r.), 164.9 (s, minor r.), 164.8 (s, major r.), 99.59 (d, major r.), 99.47 (d, minor r.), 77.99 (d), 76.44 (d), 52.67 (q), 52.40 (d, major r.), 52.19 (d, minor r.), 48.58 (t, major r.), 47.68 (t, minor r.), 36.76 (t, major r.), 35.84 (t, minor r.), 24.71 (d), 23.20 (q), 21.52 (q).

4-Amino-3-hydroxy-butyric acid methyl ester hydrochloride salt (46)

A solution containing (2*S*)-4-amino-2-hydroxy-butyric acid (2 g, 16.8 mmol) and thionyl chloride (1.84 mL, 25 mmol) in MeOH (160 ml) was stirred at room temperature overnight. Removal of the solvent afforded **46** as a pale yellow oil (2.5 g, 88% yield).

^{1}H-NMR (D_2O, 200 MHz): δ 4.18-4.04 (m, 1H), 3.53 (s, 3H), 3.01 (dd, J_1 = 13.2 Hz, J_2 = 2.6 Hz, 1H), 2.87-2.72 (m, 1H), 2.59-2.33 (m, 2H); ^{13}C-NMR (D_2O, 200 MHz): δ 172.9 (s), 64.40 (d), 52.49 (q), 43.90 (t), 39.26 (t); MS *m/z* 134 (0.75), 56 (100).

(3*R*/*S*)-3-Hydroxy-4-{(2*S*)-4-methyl-2-[(1*R*, 5*S*, 7*S*)-2-oxo-7-(R, R')-6,8-dioxa-3-aza-bicyclo[3.2.1]oct-3-yl]-pentanoylamino}-butyric acid methyl ester (48)

To a solution of **46** (37 mg, 0.22 mmol) in DCM (4 ml) were added, under N_2 atmosphere and at 0°C, PyBROP (102 mg, 0.22 mmol), carboxylic acid **47** (0.22 mmol) and DIPEA (85 μl, 0.5 mmol). The resulting solution was allowed to reach room temperature and was stirred overnight.

The reaction mixture was then washed with a saturated solution of $NaHCO_3$, aqueous 5% $KHSO_4$, brine and dried over Na_2SO_4. After evaporation of the solvent the crude product was diluted in AcOEt and left for three hours at 4°C in order to let precipitate the PyBROP. Purification by flash chromatography (silica gel, AcOEt) afforded **48** as a colorless oil.

In the following NMR characterization the signals corresponding to R and R' groups are omitted. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 6.81-6.69 (m, 1H), 5.86 (d, J = 2.4 Hz, 1H), 5.14 (d, J = 2.4 Hz, 1H), 5.10-4.81 (m, 2H), 4.16-4.05 (m, 1H), 3.69 (s, 3H), 3.56-3.20 (m, 4H), 2.46 (d, J = 5.6 Hz, 2H), 1.77-1.41 (m, 3H), 0.96-0.88 (m, 6H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 172.5 (s), 170.2 (s), 168.1 (s), 165.3 (s), 99.62 (d), 78.00 (d), 67.27 (d), 53.69, 53.01 (d, 2 diastereoisomers), 52.02 (q), 48.58 (t), 47.54 (t), 38.70 (t), 36.33 (t), 24.68 (d), 23.09 (q), 22.04 (q).

4-Methyl-2-[(1*R*, 5*S*, 7*S*)-2-oxo-7-(R, R')-6,8-dioxa-3-aza-bicyclo[3.2.1]oct-3-yl]-pentanoic acid (3-butylcarbamoyl-2-hydroxy-propyl)-amide (49)

A solution containing **48** (40 mg, 0.08 mmol) and *n*-butyl amine (168 μL, 1.7 mmol) in a mixture of THF (200 μl) and two drops of H_2O is stirred at 50°C for three days. Filtration of the reaction mixture on Amberlyst 15 and further purification by column chromatography (Silica gel, DCM/MeOH 20:1) afforded **49** as a colorless oil.

In the following NMR characterization the signals corresponding to R and R' groups are omitted. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 6.81-6.68 (m, 1H), 6.41-6.22 (m, 1H), 5.90, 5.86 (s, 1H, mix of two diastereoisomers), 5.14-4.81 (m, 3H), 4.13-3.92 (m, 1H), 3.66-3.35 (m, 4H), 3.36-3.02 (m, 2H), 2.28 (d, J = 5.2 Hz, 2H), 1.88-1.20 (m, 7H), 0.97-0.87 (m, 9H); ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 171.5 (s), 170.2 (s), 168.0 (s), 164.8 (s), 99.58 (d), 77.95 (d), 67.94 (d), 54.10, 53.87 (d, 2 diastereoisomers), 47.57 (t), 44.55 (t), 39.44 (t), 36.39 (t), 34.92 (t), 31.60 (t), 24.86 (d), 23.10 (q), 22.03 (q), 20.24 (t), 13.92 (q).

Publications

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  "Synthesis of Bicyclic Molecular Scaffolds (BTAa): an Investigation towards New Selective MMP-12 Inhibitors"
  Bioorg. Med. Chem., **2006**, *14*, 7392-7403

- Lalli, C.; Trabocchi, A.; Guarna, F.; Mannino, C.; Guarna, A.
  "Synthesis of a Bicyclic Proline Analogue from L-Ascorbic Acid"
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Synthesis of bicyclic molecular scaffolds (BTAa): An investigation towards new selective MMP-12 inhibitors

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Abstract—Starting from 3-aza-6,8-dioxa-bicyclo[3.2.1]octane scaffold (BTAa) a virtual library of molecules was generated and screened in silico against the crystal structure of the Human Macrophage Metalloelastase (MMP-12). The molecules obtaining high score were synthesized and the affinity for the catalytic domain of MMP-12 was experimentally proved by NMR experiments. A BTAa scaffold **20** having a *N*-hydroxyurea group in position 3 and a *p*-phenylbenzylcarboxy amide in position 7 showed a fair inhibition potency (IC_{50} = 149 μM) for MMP-12 and some selectivity towards five different MMPs. These results, taken together with the X-ray structure of the adduct between MMP-12, the inhibitor **20** and the acetohydroxamic acid (AHA), suggest that bicyclic scaffold derivatives may be exploited for the design of new selective matrix metalloproteinase inhibitors (MMPIs).

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent, calcium containing, endopeptidases involved in extracellular matrix degradation and, consequently, they play a crucial role in physiological processes such as tissue remodelling and healing of wounds.[1] As the upregulation of MMPs is involved in many inflammatory, malignant and degenerative diseases,[2] attempts to design and develop inhibitors that may modulate their regulation have become of great interest.[3–5]

The main requirement for a small molecule to be an effective metalloproteinase inhibitor (MMPI) is the presence of both a functional group capable of chelating the active-site zinc ion and a lipophilic residue able to fit into the S_1' pocket.[4] Hydroxamic acid is the most popular zinc binding group (ZBG) for MMPIs even if some authors report that it is fast metabolized in vivo[6,7] and it contributes to increase toxicity due to its low metal binding selectivity.[8]

The inhibitors designed up to date usually bind the active site on the catalytic domain providing nanomolar dissociation constants.[3,4] However, several inhibitors exhibit a poor selectivity as a consequence of the high structural similarity among the members of the MMP family.[9] Even if the low specificity does not prevent the use in vivo, it raises a lot of side effects and it also limits the dose that may be daily administered.[10,11] As a consequence, the search of new potent and selective MMP inhibitors still represents an important pharmaceutical target.

Even if the expensive high-throughput screening of small molecule libraries is one of the most popular approaches in pharmaceutical research, alternative strategies, based on docking calculation and rational drug design, may be adopted if the 3D structure of the target is solved.[12–14]

A new class of molecular heterocyclic scaffolds (BTAa) based on 3-aza-6,8-dioxa-bicyclo[3.2.1]octane skeleton,[15] which can be decorated with a ZBG and a

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lipophilic residue, was chosen to design selective MMP-12 inhibitors (Fig. 1).

The main feature of these scaffolds is their tridimensional bicyclic skeleton generated by combination of sugar and amino acid derivatives. Their synthesis includes only few steps starting from commercially available enantiopure precursors and a stereochemistry control can be accomplished in each step of the synthesis. As dipeptide isosteres, BTAas are compounds with potential biological activity and their use as central core of pharmaceutical targets is convenient as a high number of positions can be functionalized leading to molecular diversity. Limited flexibility can be advantageous to correctly direct the pharmacophore side chains and it is also useful for computational analysis. In addition, as amino acids, they have full compatibility with the conditions required for solid-phase synthesis.[15,16]

All these properties, together with fair solubility and low molecular weight, were considered in scaffold selection.

Diversification of BTAas by easy decoration with different functional groups may generate virtual libraries which may be screened in silico in order to identify new hit compounds for further lead discovery. Even if the binding energy provided by docking programs does not reflect exactly the real binding affinity, its value allows to score the docking results in order to identify a class of potential ligand.[17]

Moreover, the experimental assessment of the affinity of few selected molecules outlined by docking results gives the chance to correlate binding energies and binding constants improving the prediction reliability. A combined use of virtual screening and experimental determination of the binding affinity suggested that the BTAas may represent a new type of scaffolds to design a new class of MMPIs.

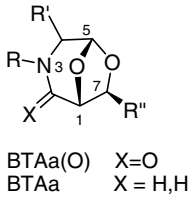


Figure 1.

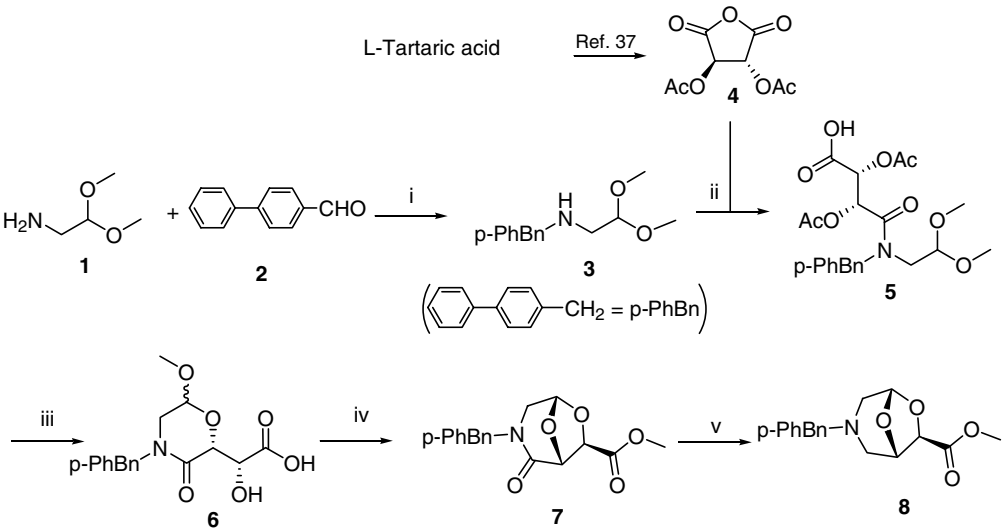
2. Chemistry

The synthesis of 3-biphenyl-4-ylmethyl-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid methyl ester (*p*-PhBn-BTGOMe) (**8**) was carried out following a procedure previously reported for similar compounds and is briefly reported below Scheme 1.[12]

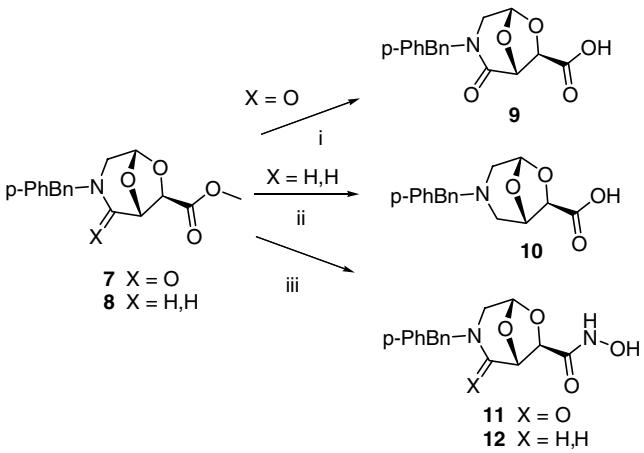
Reductive alkylation of aminoacetal **1** with biphenyl-4-carbaldehyde (**2**) and $NaBH_4$ afforded in good yield the aryl aminoacetal **3** which was subsequently converted into the amide **5** by treatment with (*R*,*R*) tartaric anhydride[18] **4**. Crude **5** was treated with thionyl chloride in MeOH affording cyclic acetal **6**, which was submitted to the acid-catalyzed cyclization to give **7** in 40% yield over the four steps. Reduction of amide **7** with $BH_3{\cdot}Me_2S$ furnished the corresponding amine **8** in 70% yield (Scheme 1).

Basic hydrolysis of **7** and acid-catalyzed hydrolysis of **8** gave, respectively, the free acids **9** and **10** in good yields.

A peculiar feature of BTAas is the demonstrated high reactivity of their methyl ester function towards amines, although simple esters are usually not very reactive towards direct aminolysis.[19] Owing to this, hydroxamic acids **11** and **12** were easily prepared by mixing the scaffold



Scheme 1. Reagents and conditions: (i) $NaBH_4$, MeOH, 25 °C, 16 h; (ii) CH_2Cl_2, 25 °C, 16 h; (iii) $SOCl_2$, MeOH, 60 °C, 2 h; (iv) H_2SO_4/SiO_2, toluene, reflux, 20 min, 41% (over the four steps); (v) $BH_3{\cdot}Me_2S$, THF, 25 °C, 16 h, 70%.



Scheme 2. Reagents and conditions: (i) LiOH, MeOH/THF, 25 °C, 12 h, 80%; (ii) HCl 4 M, 25 °C, 12 h, 90%; (iii) $NH_2OH{\cdot}HCl$, NEt_3, $CHCl_3$, 25 °C, 48 h, 21% (**11**), 26% (**12**).

with an excess of hydroxylamine hydrochloride (5 equiv) and NEt_3 in $CHCl_3$ at room temperature (Scheme 2).

To prepare the model compound **15** having a *N*-hydroxyurea as zinc-binding group, piperidine **13** was treated with triphosgene and then with benzyl-hydroxylamine and NEt_3 affording compound **14**. The debenzylated product **15** was readily obtained by mild hydrogenolytic cleavage of the protecting group over 5% Pd/C at 1 atm (Scheme 3).[20]

A new BTAa scaffold **20** having the *N*-hydroxyurea group in position 3 and the *p*-phenylbenzylcarboxy amide in position 7 was then prepared. Due to the high reactivity of ester **16**, the biphenyl derivative **17** was easily and quantitatively prepared by stirring the starting material at 60 °C with a neat excess of amine. Debenzylation was carried out by refluxing a solution of **17** in MeOH in the presence of ammonium formate and catalytic Pd/C. Then, compound **18** was activated with carbonyldiimidazole (CDI) and treated with *O*-benzylhydroxylamine, as a free base, providing the substituted *N*-hydroxyurea **19** in 60% yield after crystallization.[21] Hydrogenolytic cleavage of the benzyl group over 5% Pd/C afforded the desired final compound **20** (Scheme 4).

3. Results and discussion

The availability of high-resolution structure of the MMP-12 catalytic domain[22,23] provided the possibility to employ a structure-based approach to design and screen a virtual library.

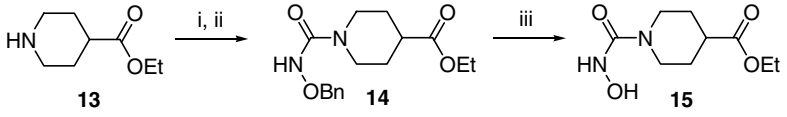
A biphenylic moiety and a hydroxamic (or carboxylic) acid were chosen as good substituents[24] to be introduced in different position of BTAa and hundreds of structures were so generated and tested in silico for their affinity to the catalytic domain of MMP-12.

The hits with the highest score were selected and, among them, compounds **9-12** were synthesized due to their higher feasibility. In this class of molecules the scaffold supports the biphenylic moiety in front of the ZBG. The Autodock binding model predicted a deep insertion of the biphenyl group into the S_1' cavity though the coordination geometry of the ZBG appeared not to be optimized.

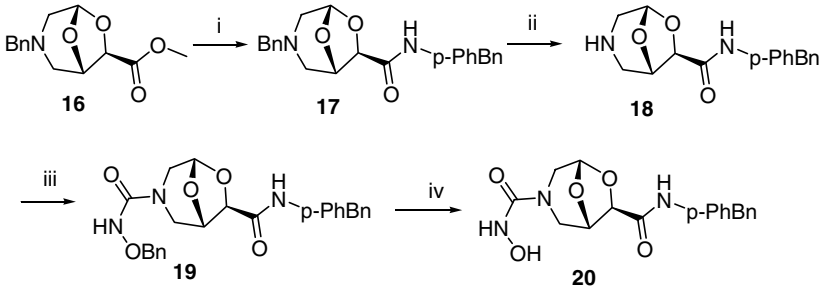
Dissociation constants (K_D) of compounds **9**–**12** were calculated through ^{15}N–^{1}H HSQC experiments.[25] In addition, compounds **21**–**24**, already present in our chemical library, were submitted to the same experiments in order to compare the results and support the docking model.

Moreover, the inhibitory activity (IC_{50}) of compounds **9**–**12** was measured by enzymatic assays.[26]

The values of K_D and IC_{50} for the tested compounds **9**–**12** and **20**–**24** are reported in Table 1.

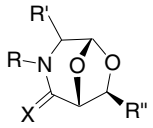


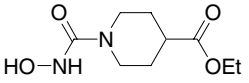
Scheme 3. Reagents and conditions: (i) $(CCl_3O)_2CO$, CH_2Cl_2, from 0 °C to 25 °C, 12 h; (ii) Bn–$ONH_2{\cdot}HCl$, NEt_3, CH_2Cl_2, 25 °C, 12 h, 40%; (iii) H_2 (1 atm), 5% Pd/C, AcOEt, 25 °C, 12 h, quantitative.



Scheme 4. Reagents and conditions: (i) *p*-PhBn–NH_2, 60 °C, 12 h, 93%; (ii) HCO_2NH_4, 10% Pd/C, MeOH, reflux, 2 h, 64%; (iii) $BnONH_2$, CDI, THF, 25 °C, 12 h, 60%; (iv) H_2 (1 atm), 5% Pd/C, THF, 25 °C, 12 h, 68%.

Table 1. Calculated MMP-12 binding affinity (K_D) and inhibition potency (IC_{50})



Compound	X	R	R′	R″	MMP-12 K_D[c]	MMP-12 IC_{50}[d] (μM)
9	O	*p*-PhBn	H	COOH	⩾1 mM	954
10	H, H	*p*-PhBn	H	COOH	⩾1 mM	835
11	O	*p*-PhBn	H	CONHOH	⩾500 μM	425
12	H, H	*p*-PhBn	H	CONHOH	⩾500 μM	399
20	H, H	HONHCO	H	CONH–*p*-PhBn	154 μM	149
21[a]	O	Bn	H	COOH	⩾10 mM	—
22[a]	H, H	Bn	H	COOH	⩾10 mM	—
23[a]	O	Bn	H	CONHOH	⩾10 mM	—
24[a]	H, H	*p*-PhBn	COOH	H	not detec.[b]	—
		 **15**			3.4 mM	—

[a] Compounds previously described.[15,29,30]
[b] No detectable interaction found by NMR.
[c] Dissociation constants measured through ^{15}N–^{1}H HSQC NMR experiments.
[d] Inhibition potency measured by fluorescence enzymatic assays.

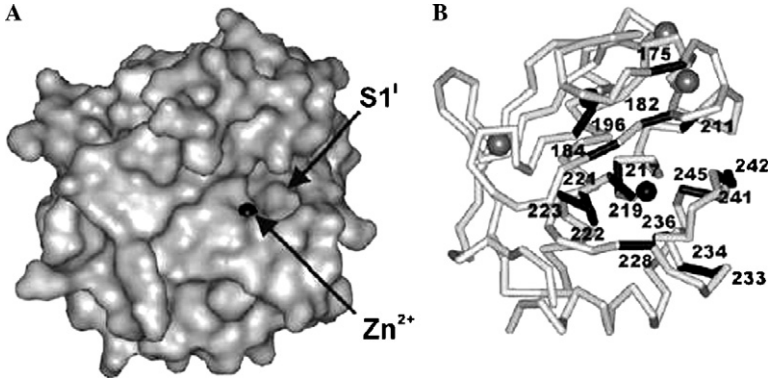


Figure 2. (A) Surface model of the MMP-12 showing the catalytic zinc and the S_1' pocket. (B) Residues of the MMP-12 affected by chemical shift perturbation upon the addition of **12**.

Since the identification of the amino acids involved in the binding may be crucial to prove a direct interaction with the catalytic site and, consequently, for the optimization of the process, NMR-based strategies were also used to localize the binding site on the target.[27] The identification of the binding site was easily performed analyzing the protein resonance chemical shifts in ^{1}H–^{15}N HSQC spectra, recorded either in presence or in absence of the ligand.[28]

The pattern of the shifts was similar for all ligands. Several residues forming the S_1' cavity, one or more zinc binding histidines and the neighbouring amino acids are strongly influenced by the presence of this class of molecules. As example, a ribbon representation of MMP-12 is shown in Figure 2. The NHs showing significant chemical shift perturbation upon the addition of compound **12** are highlighted. The affected amino acids nicely define the ligand binding site that is the same for all the designed compounds in agreement with the docking results. Only few differences were found among the members of this class. In particular, the NH cross-peak of His218 was only weakly shifted by lactams **9** and **11** with respect to compound **12**.

The analysis of the results summarized in Table 1 suggests that the bicyclic skeleton of BTAa should be compatible with the metalloelastase catalytic domain, but the activity is strongly modulated by the type and position of the substituents. Hydroxamic acids **11**, **12** (IC_{50} = 425 μM, 399 μM, respectively) are more potent than the carboxylic ones **9**, **10** (IC_{50} = 954 μM, 835 μM, respectively). The affinity observed within this series seemed to be highly dependent on the presence of the biphenylic moiety: the replacement of the biphenylmethyl with a benzylic-protecting group resulted in a loss of binding affinity, so compounds **21** and **22** ($K_D \geqslant 10$ mM) are almost inactive; and the introduction of the hydroxamic moiety in position 7 did not restore the activity (see compound **23**). Otherwise, the presence of the ZBG and the lipophilic residue in vicinal position decreases the binding affinity as demonstrated

by compound **24** for which no detectable interactions were found by NMR.

With these results in our hands we decided to focus our efforts on the design of new derivatives with improved affinity. A deeper penetration in the S_1' cavity was possible moving the biphenyl group quite far from the scaffold. Due to the high reactivity of the BTAa methyl ester, it was synthetically very easy to introduce the biphenylic moiety in 7 through an amide bond. As a consequence, the best anchoring point for the ZBG resulted in the N-3 giving rise to a *N*-hydroxyurea (compound **20**). The use of this functional group as metal chelator in MMP inhibitors has been previously reported[6] and recently validated by a X-ray structure.[31]

In order to determine the affinity of the *N*-hydroxyurea moiety, the model compound **15** was tested in silico and synthesized (see Scheme 3).

The in silico model suggested that the interaction of **15** with the active site is limited to the catalytic zinc and to the entrance into the S_1' subsite. The structure of the adduct shows the same coordination scheme of the hydroxamic acid which is able both to chelate the active-site zinc ion and to provide a hydrogen bond interaction with the enzyme backbone. The protonated oxygen atom (O4) is involved in a strong hydrogen bond with carboxylate Oε2 of Glu219, while the NH shows weaker electrostatic interaction with the Ala181 carbonyl oxygen.

To determine the binding affinity for the MMP-12 catalytic domain, we analyzed the alteration of the chemical shifts induced on 2D ^1H–^{15}N HSQC upon the titration with the *N*-hydroxyurea derivative **15**. The fit of the experimental data provided a dissociation constant of 3.4 mM. The binding mode predicted in silico is supported by the pattern of resonance frequencies shifted as a consequence of the interaction. The NH signal corresponding to His218 is strongly affected by millimolar concentration of the ligand (Fig. 3), while only few residues forming the S_1' cavity are weakly influenced.

Since the metal binding capability of the *N*-hydroxyurea was confirmed, the new scaffold **20** was synthesized. (see Scheme 4)

The ^1H–^{15}N HSQC spectra for **20** showed a pattern of shifts similar to compounds **9**–**12** indicating for all these molecules the same binding site (Fig. 4).

A different effect on the zinc binding histidine with respect to the *N*-hydroxyurea prototype is probably present since the shift perturbation involved mainly His222 instead of His218. The fit of $\Delta\delta$ as a function of the ligand concentration provided a K_D of 154 μM, in good agreement with the IC_{50} value of 149 μM obtained by the enzymatic assay (Fig. 4).

To probe the selectivity towards MMP-12, *N*-hydroxyurea **20** was also tested by fluorescence enzymatic experiments for the inhibition of collagenases (MMP-1, MMP-8, MMP-13), matrilysin 1 (MMP-7) and stromelysin 2 (MMP-10) and the corresponding data are summarized in Table 2. Compound **20** is a fairly selective MMP-12 inhibitor since it possesses limited activity against the other MMPs especially the MMP-1 and MMP-7. These two enzymes differ from other MMP family members since they have a relatively small S_1' pocket which presumably cannot accommodate the large biaryl substituent.

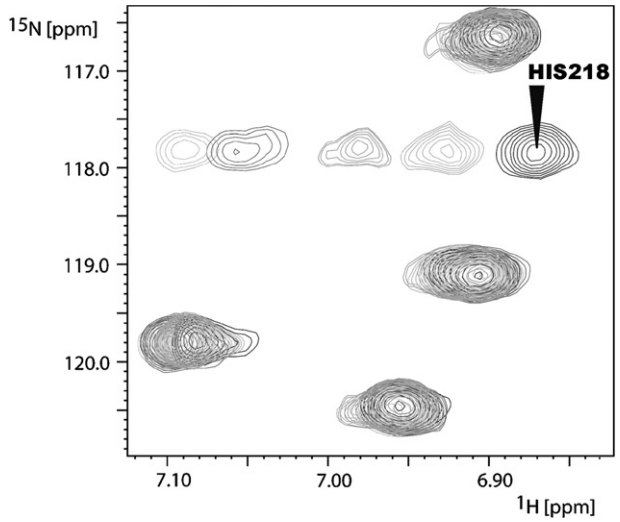


Figure 3. Expansion of ^1H–^{15}N HSQC spectrum showing the shift of the His218 backbone NH upon the addition of increasing concentration of compound **15**.

Although compound **20** exhibits an improved affinity towards MMP-12, nevertheless the micromolar value of K_D demonstrates that all the planned interactions are not optimized. The X-ray structure of the MMP-12–**20** complex (PDB code: 2HU6) provided a direct inspection on ligand–protein interactions (Fig. 5A). As correctly predicted by the NMR studies the molecule binds the metalloelastase at the catalytic site, fitting the biaryl moiety into the S_1' pocket. The electron density of the inhibitor is well defined because it is held in place by two hydrogen bonds with Pro238 and Leu181 of the enzyme backbone. However, the contribution of the hydroxyurea to the binding is absolutely negligible since it points towards the exterior of the active site and does not interact with the catalytic zinc ion.

Noteworthy, the zinc ion is coordinated by the weak inhibitor acetohydroxamic acid (AHA) present, as stabilizer, in the crystallization buffer. This would suggest that the lack of interaction between the hydroxyurea and the catalytic zinc, evidenced in the crystal structure, is due to the competition with the high concentration of AHA.

In order to investigate this point, an energy minimization of the ligand was performed after removing AHA from the PDB and blocking the biaryl group in the crystal structure conformation. The AMBER-8 minimized model indicates that the hydroxyurea function could

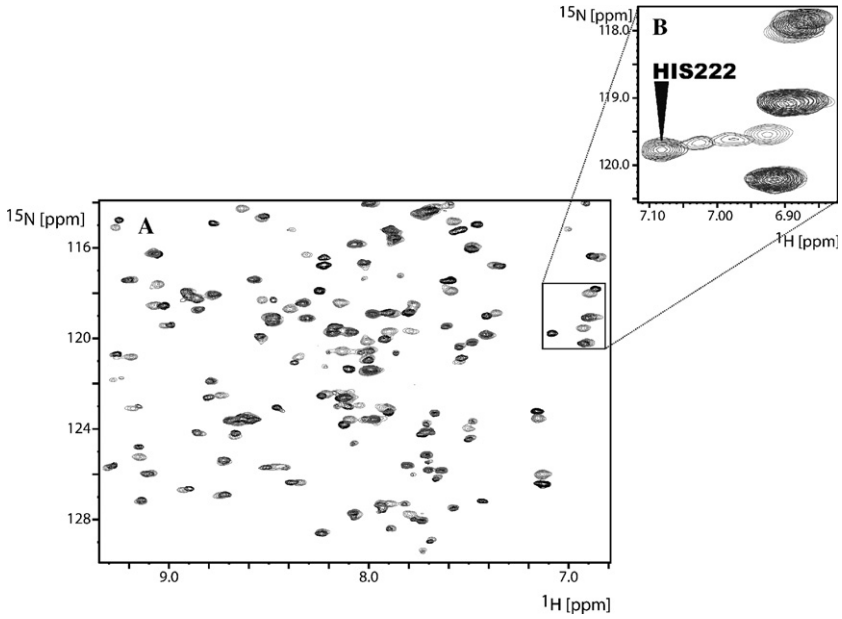


Figure 4. (A) ^{1}H–^{15}N HSQC spectrum of MMP-12 catalytic domain in absence (black) and in presence (light grey) of compound **20** (500 μM). (B) Expansion of ^{1}H–^{15}N HSQC spectrum showing the shift of the His222 backbone NH upon the addition of increasing concentration of compound **20**.

Table 2. MMP enzyme inhibition potency of compound **20** measured by enzymatic assay

Compound	IC_{50} (μM)					
	MMP-1	MMP-7	MMP-8	MMP-10	MMP-12	MMP-13
20	1180	1510	513	865	149	778

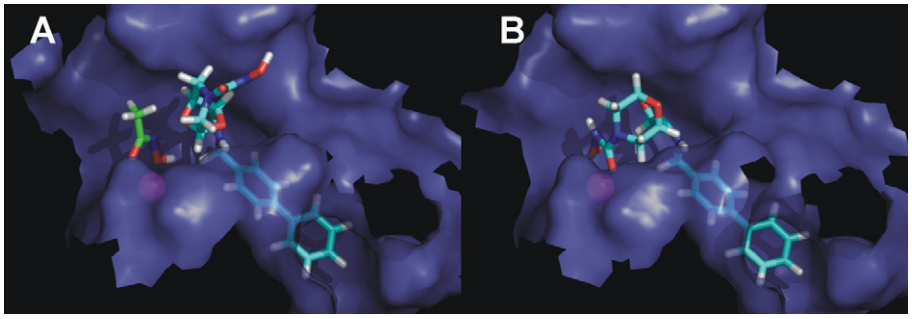


Figure 5. (A) Crystal structure of MMP-12–**20** adduct; the zinc ion is coordinated by AHA (Green). (B) AMBER-8-minimized model of MMP-12–**20** adduct.

coordinate the zinc ion, even if the hydrogen bond interactions with the zinc binding site are not well defined as in the case of AHA (Fig. 5B).

Therefore, these data suggest that the anchoring point and/or the length of the ZBG are not optimized and further studies have to be carried out in order to design a nanomolar inhibitor.

4. Conclusion

New compounds with affinity for the catalytic domain of the MMP-12 were designed using an integrated approach based on virtual screening and on NMR analysis. The molecules are built around a BTAa scaffold functionalized with a biphenyl moiety and with a ZBG.

The adducts provided by docking calculation enabled the identification of the catalytic pocket of MMP-12 as the binding site for this class of compounds. The inspection of the residues showing a significant chemical shift perturbation supports this picture, with the lipophilic moiety fitted into S_1' cavity and the bicyclic skeleton located outside, in front of the catalytic zinc.

Among the synthesized compounds, the scaffold **20** having the *N*-hydroxyurea group in position 3 and the *p*-phenylbenzylcarboxy amide in position 7 showed an improved affinity (K_D = 154 μM and IC_{50} 149 μM) for MMP-12 and some selectivity towards the other MMPs.

The X-ray structure of the catalytic domain of Human Macrophage Metalloelastase in the presence of hydroxyurea derivative **20** gave more insight into the ligand–enzyme interaction and appeared as a new starting point for further MMP-12 inhibitors' designing.

New fragments may be easily inserted in the bicyclic scaffold to better interact with the enzyme backbone and to selectively accommodate into the binding pocket of MMP-12. Therefore, the synthetic versatility of BTAas, taken together with dissociation constant in micromolar range and fair selectivity, candidate these molecules as new compound guides to develop more potent and selective inhibitors for MMP-12.

5. Methods

Docking calculations were performed using the Lamarckian genetic algorithm (LGA) of the program Autodock 3.0[32] which, using principles of natural evolution mutations and cross-over, probes the designed molecules on the three-dimensional structure of the target macromolecule. In this work, the crystal structure of the catalytic domain of MMP-12 (PDB code: 1RMZ), processed by AutoDockTools, was used as target protein. The three-dimensional structures of the designed ligands, generated by Chem3D Pro program, were minimized by semiempirical calculations and the Gasteiger-Marsili charges[33] assigned by the program Babel. In order to include all the active site, a box of $19.75 \times 19.75 \times 19.75$ Å^3, centred near the catalytic zinc and with a grid spacing of 0.275 Å, was selected as docking space. A total of 50 runs were performed for each ligand and the results were ranked according to the docking energy.

The cDNA, encoding the fragment Gly106-Gly263 (F171D mutant)[34] of the macrophage metalloelastase, was cloned into the pET21 vector (Novagen) using *Nde*I and *Bam*HI as restriction enzymes and then transfected into *E. coli* strain BL21 Codon Plus cells. Uniform ^{15}N-labelled protein was expressed by induction with 0.5 mM IPTG at 37 °C for 4 h in M9 minimal media containing 15 mM $(^{15}NH_4)_2SO_4$.

The inclusion bodies, containing the protein, were solubilized in a buffered solution with 20 mM Tris–HCl and 8 M urea at pH 8. The protein was then purified with a size-exclusion chromatography (Pharmacia HiLoad Superdex 75 16/60) in 6 M urea and 50 mM sodium acetate. A second step of purification was performed on cation exchange column Mono-S (Pharmacia) using a linear gradient of NaCl up to 0.5 M.

The protein was refolded by using a multi-step dialysis against solution containing 50 mM Tris–HCl (pH 7.2), 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.3 M NaCl and decreasing concentrations of urea (from 4 M up to 2 M). The last two dialyses were performed against solution containing 20 mM Tris–HCl (pH 7.2), 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.3 M NaCl and 200 mM of hydroxamic acid (AHA). The catalytic domains of MMP-1 (Val101-Pro269), MMP-7 (Tyr100-Lys272), MMP-8 (Met100-Gly262), MMP-10 (Phe99-Gly263, F170N mutant), MMP-13 (Tyr104-Pro269) were expressed, purified and refolded according to the protocol already described for MMP-12, with minor modifications.

^{1}H–^{15}N HSQC experiments, implemented with the sensitivity enhancement scheme,[35] were recorded at 298 K on a Bruker DRX 700 operating at proton nominal frequency of 700.21 MHz. The NMR experiments were performed on samples containing 0.1 mM of ^{15}N-enriched MMP-12 in 10 mM Tris–HCl buffer, 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.3 M NaCl and 0.2 M acetohydroxamic acid at pH 7.2.

The compounds were evaluated for their ability to inhibit the hydrolysis of fluorescence-quenched peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Biomol, Inc.). The assays were performed in 50 mM HEPES buffer, containing 10 mM $CaCl_2$, 0.05% Brij-35, at pH 7, using 1 nM of proteolytic enzyme (catalytic domains of MMP-1, MMP-7, MMP-8, MMP-10, MMP-12, MMP-13) and 1 μM of peptide. The enzyme was incubated at 25 °C with increasing concentration of inhibitor and the fluorescence ($excitation_{max}$ 328 nm; $emission_{max}$ 393 nm) was measured for 3 min after the addition of the substrate using a Varian Eclipse fluorimeter. Fitting of rates as a function of inhibitor concentration provided IC_{50} values (see Table 1). The inhibitor *N*-isobutyl-*N*-[4-methoxyphenylsulfonyl]glycyl hydroxamic acid (Biomol, Inc.) was used as control.

Crystals of human MMP-12, already containing AHA from the refolding process, grew at 20 °C from a 0.1 M Tris–HCl, 30% PEG 6000, 200 mM AHA, 1.0 M $LiCl_2$ solution at pH 8.0 using the vapour diffusion technique. The final protein concentration was about 10 mg/ml. Soaking procedure was implemented in order to allow the inhibitor binding; **20** was added in the powdered form directly into the drop using a needle and was left incubating for a few days. The data were measured exploiting synchrotron radiation at the beamline ID23-1 (ESRF, Grenoble, France). The dataset was collected at 100 K and the crystal used for data collection was cryo-cooled without any cryo-protectant treatment. The crystal diffracted up to 1.3 Å resolution and belongs to space group *C*2 (a = 51.25 Å, b = 60.18 Å, c = 53.97 Å, $\alpha = \gamma = 90°$, $\beta = 114.59°$) with one molecule in the asymmetric unit, a solvent content of about 50% and a mosaicity of 0.7°. The data were processed in all cases using the program MOSFLM[36] and scaled using the program SCALA[37] with the TAILS and SECONDARY corrections on (the latter restrained with a TIE SURFACE command) to achieve an empirical absorption correction.

Table 3 shows the data collection and processing statistics for all datasets. The structure was solved using the molecular replacement technique; the model used was that of the MMP-12–AHA adduct (1Y93) from where the inhibitor, all the water molecules and ions were

Table 3. Data collection and anisotropic refinement statistics[a]

Space group	*C*2
Cell dimensions (Å,°)	A = 51.25, b = 60.18, c = 53.97, β = 114.59
Resolution (Å)	49.10–1.32
Unique reflections	34825 (5291)
Overall completeness (%)	99.0 (98.8)
$R_{sym(\%)}$	5.3 (27.6)
Multiplicity	3.1 (2.9)
$I/(\sigma I)$	8.6 (2.5)
Wilson plot B-factor (Å^2)	10.55
R_{cryst}/R_{free} (%)	16.2/18.7
Protein atoms (excluding hydrogens)	1238
Ions	5
Ligand atoms	33
Water molecules	300
rmsd bond lengths (Å)	0.006
rmsd bond angles (°)	1.0
Mean B-factor (Å^2)	13.90

[a] The numbers in parentheses refer to values in the highest resolution shell.

omitted. The correct orientation and translation of the molecule within the crystallographic unit cell was determined with standard Patterson search techniques[38,39] as implemented in the program MOLREP.[40,41] The anisotropic refinement was carried out using REFMAC5.[42] In between the refinement cycles the model was subjected to manual rebuilding by using XtalView.[43] The same program was used to model the inhibitors. Water molecules were added by using the standard procedures within the ARP/WARP suite.[44] The stereochemical quality of the refined models was assessed using the program Procheck.[45] The Ramachandran plot is of very good quality.

6. Experimental

Melting points are uncorrected and were measured on microscope RCH Kofler apparatus. Chromatographic purifications were recorded with silica gel 60 (0.040–0.063 mm), unless otherwise stated, using flash column techniques; all TLC development was performed on silica gel coated (Merck Silica gel 60 F_{254}, 0.25 mm) sheets. IR spectra were recorded on Perkin-Elmer 881 spectrophotometer. ^{1}H and ^{13}C NMR spectral analyses were obtained on a Varian Gemini 200 (200 MHz for ^{1}H and 50.33 MHz for ^{13}C) instrument or a Varian Mercury 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) instrument using tetramethylsilane as internal standard and the chemical shifts were reported in δ (ppm) units. EI mass spectral analyses were carried out at 70 eV ionizing voltage by direct introduction on a QMD 1000 Carlo Erba instrument, whereas ESI-MS spectra were carried out on LTQ Thermo Finnigan instrument. Microanalyses were carried out on Perkin Elmer 240 C elemental analyser. Optical rotation measurements were performed on JASCO DIP-370 digital polarimeter. All reactions requiring anhydrous conditions were carried out under N_2 atmosphere and were performed in oven-dried glassware.

Preparation of acid silica gel (SiO$_2$/H$_2$SO$_4$): SiO_2 (29 g) was suspended in CH_2Cl_2 (400 ml) with 12 g of H_2SO_4 and left under vigorous magnetic stirring for 40 min. The suspension was then concentrated at low pressure.

6.1. Biphenyl-4-yl-methyl-(2,2-dimethoxy-ethyl)-amine (3)

A solution containing 2,2-dimethoxy-ethylamine (14.4 g, 137 mmol) and biphenyl-4-carbaldehyde (25 g, 137 mmol) in MeOH (300 ml) was stirred at room temperature for 2.5 h. $NaBH_4$ was then slowly added at 0 °C and the reaction mixture was stirred at room temperature overnight. Once the reaction was completed MeOH was evaporated in vacuo and the crude residue was suspended in ethyl acetate, washed with water and dried over Na_2SO_4. The resulting product was used in next step without further purification. Purification by column chromatography using ethyl acetate/petroleum ether (1:1) as eluent afforded the pure **3** as a colourless oil.

^{1}H NMR ($CDCl_3$): δ 7.62–7.26 (m, 9H), 4.53 (t, 1H, J = 5.5 Hz), 3.86 (s, 2H), 3.39 (s, 6H), 2.80 (d, 2H, J = 5.6 Hz); ^{13}C ($CDCl_3$): δ 140.9, 139.9, 139.1 (s), 128.7–127.0 (d), 104.0 (d), 54.10 (t), 53.68 (t), 50.70 (q); MS m/z 271 (M^+, 15), 167 (100); IR ($CHCl_3$) 3323, 2829 cm^{-1}; Anal. Calcd for $C_{16}H_{19}NO_2$ (257.1): C, 74.68; H, 7.44; N, 5.44. Found: C, 74.75; H, 7.52; N, 5.65.

6.2. (2*R*,6*R*/*S*)-(4-Biphenyl-4-yl-methyl-6-methoxy-3-oxo-morpholin-2-yl)-(*R*)-hydroxyacetic acid methyl ester (6)

To a suspension of (*R*,*R*)-2,3-di-*O*-acetyltartaric anhydride (**4**) (30 g, 137 mmol) in dry DCM (120 ml) was added, at 0 °C and under N_2 atmosphere, a solution of **3** (37 g, 137 mmol) in dry DCM (60 ml). The reaction mixture was stirred at room temperature overnight. After evaporation of the solvent, the crude product was dissolved in MeOH (230 ml) and thionyl chloride (8.5 ml, 116 mmol) was added dropwise at 0 °C. The mixture was then allowed to reach 60 °C and stirred for 2 h. The solvent was removed and the crude product was isolated as a yellow oil and used without further purification in the next step. Purification of the crude product by column chromatography (ethyl acetate/petroleum ether 2:1) afforded **6** as a white solid.

Mp 115–120 °C; $[\alpha]_D^{26}$ +111.1 (c 1.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.60–7.26 (m, 9H), 4.96 (s, 1H), 4.87 (s, 1H), 4.84 (d, 1H, J = 14 Hz), 4.64 (s, 1H), 4.54 (d, 1H, J = 14 Hz), 3.85 (s, 3H), 3.58 (dd, 1H, J_1 = 14 Hz, J_2 = 3.2 Hz), 3.39 (s, 3H), 3.16 (d, 1H, J = 14 Hz); ^{13}C NMR ($CDCl_3$): δ 172.4 (s), 165.4 (s), 140.6, 140.4, 134.5 (s), 128.7, 128.2, 127.4, 127.2, 127.0 (d), 95.95 (d), 72.28 (d), 71.60 (d), 55.15 (q), 52.89 (q), 49.65 (t), 49.55 (t); MS m/z 385 (M^+, 58), 167 (100); IR ($CHCl_3$) 3545, 1745, 1654 cm^{-1}; Anal. Calcd for $C_{21}H_{23}NO_6$ (385.2): C, 65.44; H, 6.02; N, 3.63. Found: C, 65.03; H, 6.22; N, 3.27.

6.3. (1*S*,5*S*,7*R*)-3-Biphenyl-4-ylmethyl-2-oxo-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid methyl ester (7)

A solution of **6** (52 g, 137 mmol) in toluene (200 ml) was quickly added to a refluxing suspension of H_2SO_4/SiO_2 (29 g) in toluene (400 ml). The mixture was allowed to react for 20 min and one-third of the solvent was distilled off. The hot reaction mixture was filtered through a short layer of $NaHCO_3$ and, after evaporation of the solvent, the crude product was purified by column chromatography using ethyl acetate/petroleum ether (2:1) as eluent and affording **7** (20 g, 41% over four steps) as a white solid.

Mp 160–165 °C; $[\alpha]_D^{25}$ −40.5 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 7.60–7.26 (m, 9H), 5.89 (d, 1H, $J = 2.2$ Hz), 5.01 (s, 1H), 4.79 (s, 1H), 4.66–4.52 (Ab system, 2H), 3.81 (s, 3H), 3.42 (dd, 1H, $J_1 = 2.2$ Hz, $J_2 = 12$ Hz), 3.17 (d, 1H, $J = 12$ Hz); ^{13}C NMR ($CDCl_3$): δ 169.0 (s), 165.4 (s), 140.9, 140.4, 134.2 (s), 128.7, 128.4, 127.6, 127.4, 127.0 (d), 100.1 (d), 77.83 (d), 77.73 (d), 52.93 (q), 51.23 (t), 48.21 (t); MS *m*/*z* 353 (M^+, 11), 167 (100); IR ($CHCl_3$) 1756, 1674 cm^{-1}; Anal. Calcd for $C_{20}H_{19}NO_5$ (353.1): C, 67.98; H, 5.42; N, 3.96. Found: C, 67.54; H, 5.42; N, 3.69.

6.4. (1*S*,5*S*,7*R*)-3-Biphenyl-4-ylmethyl-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid methyl ester (8)

$BH_3{\cdot}SMe_2$ (0.403 ml, 4.2 mmol) was added dropwise to a cooled (0 °C) solution of **7** (1 g, 2.8 mmol) in dry THF under N_2 atmosphere and the resulting mixture was stirred at room temperature overnight. Once the reaction was completed EtOH (2 ml) and H_2O were added and the resulting mixture was washed with OEt_2. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (3:1) as eluent to afford **8** (670 mg, 70%) as a white solid.

Mp 130–134 °C; $[\alpha]_D^{24}$ −57.0 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 7.63–7.36 (m, 9H), 5.66 (s, 1H), 4.86 (s, 1H), 4.66 (s, 1H), 3.78 (s, 3H), 3.70–3.50 (AB system, 2H), 2.87 (pseudo t, 2H, $J = 11.6$ Hz), 2.56 (dd, 1H, $J_1 = 1.6$, $J_2 = 11.2$ Hz), 2.35 (d, 1H, $J = 12$ Hz); ^{13}C NMR ($CDCl_3$): δ 171.4 (s), 140.7, 140.2, 136.3 (s), 129.2, 128.7, 127.2, 127.1, 127.0 (d), 101.4 (d), 77.00 (d), 76.07 (d), 61.16 (t), 56.44 (t), 54.85 (t), 52.54 (q); MS *m*/*z* 339 (M^+, 23), 167 (100); IR ($CHCl_3$) 1753, 1733 cm^{-1}; Anal. Calcd for $C_{20}H_{21}NO_4$ (339.2): C, 70.78; H, 6.24; N, 4.13. Found: C, 70.34; H, 6.41; N, 4.00.

6.5. (1*S*,5*S*,7*R*)-3-Biphenyl-4-ylmethyl-2-oxo-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid (9)

A solution containing **7** (85 mg, 0.24 mmol) and LiOH (25 mg, 0.6 mmol) in a mixture of MeOH and THF in a 2:1 ratio (5 ml) was stirred overnight at room temperature. The reaction mixture was then acidified with 5% HCl and the organic solvents were removed in vacuo. The remaining aqueous layer was washed with DCM and the resulting organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to afford **9** (65 mg, 80%) as a white solid.

Mp 186–195 °C; ^1H NMR (DMSO): δ 7.66–7.27 (m, 9H), 5.95 (s, 1H), 4.89 (s, 1H), 4.80 (s, 1H), 4.60–4.42 (AB system, 2H), 3.38 (d, 1H, $J = 14$ Hz), 3.10 (d, 1H, $J = 14$); ^{13}C NMR (DMSO): δ 170.6 (s), 165.7 (s), 140.1, 139.7, 135.9 (s), 129.3, 128.7, 127.8, 127.4, 127.0 (d), 99.85 (d), 77.62 (d), 77.50 (d), 51.64 (t), 47.39 (t); MS *m*/*z* 339 (M^+, 17), 167 (100); Anal. Calcd for $C_{19}H_{17}NO_5$ (339.1): C, 67.25; H, 5.05; N, 4.13. Found: C, 66.86; H, 5.06; N, 4.04.

6.6. (1*S*,5*S*,7*R*)-3-Biphenyl-4-ylmethyl-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid (10)

A solution containing **8** (80 mg, 0.23 mmol) and aq. HCl 4 M (0.800 ml) was stirred overnight at room temperature. The product was then freeze-dried affording **10** (75 mg, 90%) as a white solid.

Mp 158–165 °C; ^1H NMR (DMSO): δ 11.18 (br s, 1H), 7.72–7.67 (m, 5H), 7.48–7.35 (m, 4H), 5.83 (s, 1H), 5.45 (s, 1H), 4.97 (s, 1H), 4.34 (m, 2H), 3.60–3.48 (m, 1H), 3.42–3.22 (m, 1H), 3.20–2.96 (m, 2H); ^{13}C NMR (DMSO) (carboxylic carbon not detected): δ 139.7, 132.8 (s), 129.4–127.1 (d), 101.0 (d), 75.03 (d), 74.31 (d), 59.47 (t), 53.19 (t), 52.86 (t); MS *m*/*z* 325 (M^+, 21), 167 (100); IR (KBr) 3406, 2355, 1741 cm^{-1}; Anal. Calcd for $C_{19}H_{20}ClNO_4$ (361.11): C, 63.07; H, 5.57; N, 3.87. Found: C, 62.88; H, 5.71; N, 3.75.

6.7. (1*S*,5*S*,7*R*)-3-Biphenyl-4-ylmethyl-2-oxo-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid hydroxyamide (11)

A solution containing **7** (100 mg, 0.28 mmol), hydroxylamine hydrochloride (97 mg, 1.42 mmol) and NEt_3 (236 μl, 1.7 mmol) in $CHCl_3$ (500 μl) was stirred for 5 days at room temperature. The resulting suspension was then filtered and the organic solvents were evaporated in vacuo. The crude product was purified by column chromatography, using ethyl acetate as eluent and affording **11** (21 mg, 21%) as a white solid.

Mp 197–203 °C; ^1H NMR (DMSO): δ 10.74 (br s, 1H), 9.00 (br s, 1H), 7.71–7.17 (m, 9H), 5.92 (s, 1H), 4.69 (s, 2H), 4.49 (s, 2H), 3.39 (d, 1H, $J = 12$ Hz), 3.07 (d, 1H, $J = 12$ Hz); ^{13}C NMR (DMSO): δ 165.9 (s), 165.2 (s), 140.1, 139.7, 135.9 (s), 129.3, 128.7, 127.8, 127.4, 127.0 (d), 99.84 (d), 78.84 (d), 77.46 (d), 51.60 (t), 47.41 (t); MS *m*/*z* 354 (M^+, 2), 167 (100); Anal. Calcd for $C_{19}H_{18}N_2O_5$ (354.1): C, 64.40; H, 5.12; N, 7.91. Found: C, 64.12; H, 5.42; N, 7.53.

6.8. (1*S*,5*S*,7*R*)-3-Biphenyl-4-ylmethyl-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid hydroxyamide (12)

A solution containing **8** (98 mg, 0.29 mmol), hydroxylamine hydrochloride (97 mg, 1.42 mmol) and NEt_3 (242 μl, 1.7 mmol) in $CHCl_3$ (500 μl) was stirred for 5 days at room temperature. The resulting suspension

was then filtered and the organic solvents were evaporated in vacuo. The crude product was purified by column chromatography, using ethyl acetate as eluent, affording **12** (26 mg, 26%) as a white solid.

Mp 185–189 °C; ^{1}H NMR (DMSO): δ 10.55 (s, 1H), 8.80 (s, 1H), 7.67–7.34 (m, 9H), 5.56 (s, 1H), 4.52 (s, 1H), 4.46 (s, 1H), 3.56 (s, 2H), 2.75 (pseudo t, 2H, J = 10 Hz), 2.42 (d, 1H, J = 11.4 Hz), 2.18 (d, 1H, J = 11.6 Hz); ^{13}C NMR (DMSO): δ 167.3 (s), 140.3, 137.1 (s), 129.7, 129.3, 129.2, 127.7, 127.0 (d), 100.5 (d), 76.92 (d), 76.73 (d), 60.66 (t), 56.42 (t), 55.28 (t); ESI MS *m*/*z* 341 (M+1); IR (KBr) 3317, 3146, 1683 cm^{-1}; Anal. Calcd for $C_{19}H_{20}N_2O_4$ (340.1): C, 67.05; H, 5.92; N, 8.23. Found: C, 66.66; H, 5.45; N, 8.61.

6.9. 1-Benzyloxycarbamoyl-piperidine-4-carboxylic acid ethyl ester (14)

A solution of piperidine-4-carboxylic acid ethyl ester (**13**) (300 mg, 1.91 mmol) in dry DCM (5 ml) was added to a solution of triphosgene (187 mg, 0.63 mmol) in dry DCM (4 ml) at 0 °C and under inert atmosphere. The resulting solution was allowed to reach room temperature and stirred for 1 day.

To the reaction mixture were then added *O*-benzyl-hydroxylamine hydrochloride (336 mg, 2.1 mmol) and NEt_3 (850 μl, 6.1 mmol) and the resulting solution was stirred for 2 h. The reaction mixture was then concentrated under reduced pressure, diluted with DCM, washed with H_2O and brine, dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography (Aluminium oxide, ethyl acetate/petroleum ether 2:1 to clean up impurities; ethyl acetate to isolate the final product) to afford **14** (238 mg, 40%) as a pale yellow oil.

^{1}H NMR ($CDCl_3$): δ 7.54–7.25 (br s, 5H), 7.11 (br s, 1H), 4.84 (s, 2H), 4.14 (q, 2H, J = 7.07 Hz), 3.95–3.78 (m, 2H), 3.02–2.79 (m, 2H), 2.57–2.35 (m, 1H), 1.99–1.80 (m, 2H), 1.77–1.51 (m, 2H), 1.25 (t, 3H, J = 7.11 Hz); ^{13}C NMR ($CDCl_3$): δ 174.2 (s), 158.8 (s), 135.9 (s), 129.1, 128.5, 128.5 (d), 77.94 (t), 60.61 (t), 43.32 (t), 40.79 (d), 27.70 (t), 14.17 (q); MS *m*/*z* 306 (M^+, 1), 91 (100); IR ($CHCl_3$) 1724, 1676, 1222 cm^{-1}; Anal. Calcd for $C_{16}H_{22}N_2O_4$ (306.16): C, 62.73; H, 7.24; N, 9.14. Found: C, 61.65; H, 7.74; N, 8.94.

6.10. 1-Hydroxycarbamoyl-piperidine-4-carboxylic acid ethyl ester (15)

A stirred solution of **14** (150 mg, 0.5 mmol) in AcOEt was submitted to hydrogenolysis over 10% Pd/C at 1 atm overnight. After separation of the catalyst by centrifugation, the solvent was evaporated and the crude residue washed with Et_2O affording **15** (108 mg, quantitative) as a white solid.

Mp 101–104 °C; ^{1}H NMR ($CDCl_3$): δ 6.46 (br s, 1H), 4.13 (q, 2H, J = 7.09 Hz), 3.86 (dt, 2H, J_1 = 13.45, J_2 = 3.69 Hz), 3.05–2.81 (m, 2H), 2.48 (m, 1H), 2.03–1.82 (m, 2H), 1.78–1.53 (m, 2H), 1.24 (t, 3H, J = 7.16); ^{13}C NMR ($CDCl_3$): δ 171.8 (s), 158.2 (s), 58.02 (t), 40.14 (t), 37.91 (d), 24.81 (t), 11.44 (q); ESI MS *m*/*z* 217 (M+1); IR ($CHCl_3$) 3682, 1724, 1668, 1218 cm^{-1}; Anal. Calcd for $C_9H_{16}N_2O_4$ (216.11): C, 49.99; H, 7.46; N, 12.96. Found: C, 49.55; H, 7.45; N, 12.89.

6.11. (1*S*,5*S*,7*R*)-3-Benzyl-3-aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid (biphenyl-4-ylmethyl)-amide (17)

A mixture containing BnBTGOMe (**16**) (314 mg, 1.19 mmol) and biphenyl-4-yl-methylamine (1 g, 4.6 mmol) was allowed to reach 60 °C and was stirred at the same temperature overnight. Once the reaction was completed the crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:2) as eluent and affording **17** (460 mg, 93%) as a white solid.

Mp 98–101 °C; $[\alpha]_D^{23}$ +23.7 (*c* 0.5, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 7.58–7.19 (m, 14H), 6.97 (br s, 1H), 5.59 (s, 1H), 4.79 (s, 1H), 4.69 (s, 1H), 4.65–4.37 (m (ABX system), 2H), 3.57 (m, 2H), 2.87 (d, 2H, J = 10.6 Hz), 2.55 (d, 1H, J = 11.8 Hz), 2.34 (d, 1H, J = 11 Hz); ^{13}C NMR ($CDCl_3$): δ 171.1 (s), 140.6, 140.4, 136.7 (s), 128.8, 128.7, 128.4, 128.0, 127.4, 127.3, 127.3, 127.0 (d), 101.2 (d), 77.44 (d), 77.38 (d), 61.54 (t), 56.30 (t), 55.02 (t), 42.87 (t); MS *m*/*z* 414 (M^+, 7), 91 (100); IR ($CHCl_3$) 3418, 1674, 1521, 1106 cm^{-1}; Anal. Calcd for $C_{26}H_{26}N_2O_3$ (414.19): C, 75.34; H, 6.32; N, 6.76. Found: C, 74.96; H, 6.25; N, 6.37.

6.12. (1*S*,5*S*,7*R*)-3-Aza-6,8-dioxa-bicyclo[3.2.1]octane-7-carboxylic acid (biphenyl-4-ylmethyl)-amide (18)

A solution of **17** (438 mg, 1.06 mmol) and ammonium formate (300 mg, 4.76 mmol) in MeOH (8 ml) and over 10% Pd/C was refluxed for 2 h under N_2 atmosphere. After separation of the catalyst by filtration over a pad of Celite, the solvent was evaporated to afford **18** (220 mg, 64%) as a white solid.

Mp 89–92 °C; $[\alpha]_D^{22}$ +11.8 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.59–7.26 (m, 9H), 6.98 (br s, 1H), 5.57 (s, 1H), 4.79 (s, 1H), 4.68 (s, 1H), 4.52 (m, 2H), 3.29 (d, 1H, J = 13.2 Hz), 2.98 (d, 2H, J = 13.6 Hz), 2.80 (d, 1H, J = 13.6 Hz); ^{13}C NMR ($CDCl_3$): δ 171.0 (s), 140.6, 136.7 (s), 128.7, 128.0, 127.4, 127.3, 127.0 (d), 101.1 (d), 77.42 (d), 54.21 (t), 53.84 (t), 42.86 (t); MS *m*/*z* 324 (M^+, 5), 167 (100); IR ($CHCl_3$) 3420, 1673, 1527, 1110 cm^{-1}; Anal. Calcd for $C_{19}H_{20}N_2O_3$ (324.15): C, 70.35; H, 6.21; N, 8.64. Found: C, 70.25; H, 5.91; N, 8.90.

6.13. (1*S*,5*S*,7*R*)-3-Aza-6,8-dioxa-bicyclo[3.2.1]octane-3,7-dicarboxylic acid 3-(benzyloxy-amide) 7-[(biphenyl-4-ylmethyl)-amide] (19)

O-Benzyl-hydroxylamine[16] (69 mg, 0.56 mmol) was dissolved in anhydrous THF (1.6 ml) and added dropwise to a cooled solution of carbonyldiimidazole (91 mg,

0.56 mmol) in 5 ml of dry THF under N_2 atmosphere. After being stirred for 30 min at room temperature, the resulting solution was added to the neat **18** (181 mg, 0.56 mmol) and the reaction mixture was stirred overnight. The solution was then diluted with AcOEt, washed with water and brine, dried over Na_2SO_4 and evaporated in vacuo. Crystallization at room temperature from ethyl acetate afforded the pure **19** (160 mg, 60%) as a white solid.

Mp 110–114 °C; $[\alpha]_D^{26}$ +29.6 (*c* 0.77, $CDCl_3$); ^1H NMR ($CDCl_3$): δ 7.60–7.26 (m, 14H), 6.90 (br s, 1H), 5.62 (s, 1H), 4.85 (s, 2H), 4.76 (s, 1H), 4.60–4.48 (m, 3H), 3.89 (d, 1H, $J = 12.8$ Hz), 3.70 (d, 1H, $J = 12.8$ Hz), 3.33 (d, 1H, $J = 13$ Hz), 3.04 (d, 1H, $J = 12.8$ Hz); ^{13}C NMR ($CDCl_3$): δ 169.9 (s), 159.3 (s), 140.6, 136.4, 135.5 (s), 129.0, 128.7, 128.5, 128.5, 128.0, 127.4, 127.4, 127.0 (d), 99.78 (d), 78.21 (d), 75.69 (d), 47.42 (t), 46.88 (t), 42.98 (t); MS *m/z* 473 (M^+, 2), 324 (12), 167 (100), 91 (61); IR ($CDCl_3$) 3420, 1677, 1527 cm^{-1}; Anal. Calcd for $C_{27}H_{27}N_3O_5$ (473.20): C, 68.48; H, 5.75; N, 8.87. Found: C, 68.38; H, 5.61; N, 8.92.

6.14. (1*S*,5*S*,7*R*)-3-Aza-6,8-dioxa-bicyclo[3.2.1]octane-3,7-dicarboxylic acid 7-[(biphenyl-4-ylmethyl)-amide] 3-hydroxyamide (20)

A stirred solution of **19** (120 mg, 0.25 mmol) in THF was submitted to hydrogenolysis over 10% Pd/C at 1 atm, at room temperature, overnight. After separation of the catalyst by filtration through a pad of Celite, the solvent was evaporated and the crude residue washed with Et_2O affording **20** (65 mg, 68%) as a white solid.

Mp 75–80 °C; $[\alpha]_D^{26}$ +36.6 (*c* 0.75, $CDCl_3$); ^1H NMR ($CDCl_3$): (Mixture of rotamers): δ 11.58 (br s, 1H), 8.41 (br s, 1H), 7.62–7.20 (m, 9H), 7.13–6.90 (m, 1H), 5.65 (s, 1H, minor rotamer), 5.60 (s, 1H, major rotamer), 4.78 (s, 1H, minor rotamer), 4.73 (s, 1H, major rotamer), 4.66–4.57 (m, 1H), 4.55–4.38 (m, 2H), 4.02–3.84 (m, 1H), 3.80–3.62 (m, 1H), 3.41–3.24 (m, 1H), 3.18–2.96 (m, 1H); ^{13}C NMR ($CDCl_3$): (mixture of rotamers): δ 170.8 (s, major rotamer), 170.5 (s, minor rotamer), 161.5 (s, major r.), 159.0 (s, minor r.), 140.6, 140.5, 136.5 (s), 128.8–127.0 (d), 99.79 (d), 77.22 (d, minor r.), 76.93 (d, major r.), 75.68 (d), 47.46 (t, minor r.), 46.97 (t, major r.), 46.48 (t, minor r.), 45.88 (t, major r.), 42.80 (t); MS *m/z* 383 (M^+, 0.2), 324 (7), 167 (100); IR ($CDCl_3$) 3420, 1668 cm^{-1}; Anal. Calcd for $C_{20}H_{21}N_3O_5$ (383.15): C, 62.65; H, 5.52; N, 10.96. Found: C, 62.30; H, 5.96; N, 10.40.

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References and notes

1. Visse, R.; Nagase, H. *Circ. Res.* **2003**, *92*, 827–839.
2. Mandal, M.; Mandal, A.; Das, S.; Chakraborti, T.; Sajal, C. *Mol. Cell. Biochem.* **2003**, *252*, 305–329.
3. Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. *Chem. Rev.* **1999**, *99*, 2735–2776.
4. Skiles, J. W.; Gonnella, N. C.; Jeng, A. Y. *Curr. Med. Chem.* **2004**, *11*, 2911–2977.
5. Fisher, J. F.; Mobashery, S. *Cancer Metastasis Rev.* **2006**, *25*, 115–136.
6. Summers, J. B.; Gunn, B. P.; Mazdiyasni, H.; Goetz, A. M.; Young, P. R.; Bouska, J. B.; Dyer, R. D.; Brooks, D. W.; Carter, G. W. *J. Med. Chem.* **1987**, *30*, 2121–2126.
7. Michaelides, M. R.; Dellaria, J. F.; Gong, J.; Holms, J. H.; Bouska, J. J.; Stacey, J.; Wada, C. K.; Heyman, H. R.; Curtin, M. L.; Guo, Y.; Goodfellow, C. L.; Elmore, I. B.; Albert, D. H.; Magoc, T. J.; Marcotte, P. A.; Morgan, D. W.; Davidsen, S. K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1553–1556.
8. Breuer, E.; Frant, J.; Reich, R. *Expert Opin. Ther. Patents* **2005**, *15*, 253–269.
9. Lukacova, V.; Zhang, Y. F.; Mackov, M.; Baricic, P.; Raha, S.; Calvo, J. A.; Balaz, S. *J. Biol. Chem.* **2004**, *279*, 14194–14200.
10. Coussens, L. M.; Fingleton, B.; Matrisian, L. M. *Science* **2002**, *295*, 2387–2392.
11. Pavlaki, M.; Zucker, S. *Cancer Metastasis Rev.* **2003**, *22*, 177–203.
12. Borkakoti, N. *Biochem. Soc. Trans.* **2004**, *32*, 17–20.
13. Fragai, M.; Nativi, C.; Richichi, B.; Venturi, C. *ChemBioChem* **2005**, *6*, 1345–1349.
14. Hanessian, M.; Mackay, D. B.; Moitessier, N. *J. Med. Chem.* **2001**, *44*, 3066–3073.
15. (a) Guarna, A.; Guidi, A.; Machetti, F.; Menchi, G.; Occhiato, E. G.; Scarpi, D.; Sisi, S.; Trabocchi, A. *J. Org. Chem.* **1999**, *64*, 7347–7364; (b) Trabocchi, A.; Menchi, G.; Guarna, F.; Machetti, F.; Scarpi, D.; Guarna, A. *Synlett* **2006**, 331–353.
16. Trabocchi, A.; Mancini, F.; Menchi, G.; Guarna, A. *Mol. Diversity* **2003**, *6*, 245–250.
17. Klebe, G.; Gräder, U.; Grünemberg, S.; Krämer, O.; Gohlke, O.; *Understanding Receptor–Ligand Interaction as a Prerequisite for Virtual Screening*. In *Virtual Screening for Bioactive Molecules*, H. J. Böhm, G. Schneider, Eds.; Wiley–WCH, Weinheim, Germany, 2000; pp 207–227.
18. Lucas, H. J.; Baumgarten, W. *J. Am. Chem. Soc.* **1941**, *63*, 1653–1657.
19. Machetti, F.; Bucelli, I.; Indiani, G.; Guarna, A. *Chimie* **2003**, *6*, 631–633.
20. Defoin, A.; Brouillard-Poichet, A.; Streith, J. *Helv. Chim. Acta* **1992**, *75*, 109–123.
21. Romine, J. L.; Martin, S. W.; Meanwell, N. A.; Epperson, J. R. *Synthesis* **1994**, *8*, 846–850.
22. Bertini, I.; Calderone, V.; Cosenza, M.; Fragai, M.; Lee, Y.-M.; Luchinat, C.; Mangani, S.; Terni, B.; Turano, P. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5334–5339.
23. Lang, R.; Kocourek, A.; Braun, M.; Tschesche, H.; Huber, R.; Bode, W.; Maskos, K. *J. Mol. Biol.* **2001**, *312*, 731–742.
24. Hajduk, P. J.; Sheppard, G.; Nettesheim, D. G.; Olejniczak, E. T.; Shuker, S. B.; Meadows, R. P.; Steinman, D. H.; Carrera, G. M.; Marcotte, P. A.; Severin, J.; Walter, K.; Smith, H.; Gubbins, E.; Simmer, R.; Holzman, T. F.;

Morgan, D. W.; Davidsen, S. K.; Summers, J. B.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 5818–5827.
25. Mitton-Fry, R. M.; Anderson, E. M.; Hughes, T. R.; Lundblad, V.; Wuttke, D. S. *Science* **2002**, *296*, 145–147.
26. Knight, C. G.; Willenbrock, F.; Murphy, G. *FEBS Lett.* **1992**, *296*, 263–266.
27. Pellecchia, M.; Becattini, B.; Crowell, K. J.; Fattorusso, R.; Forino, M.; Fragai, M.; Jung, D.; Mustelin, T.; Tautz, L. *Expert Opin. Ther. Targets* **2004**, *6*, 597–611.
28. Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531–1534.
29. Guarna, A.; Bucelli, I.; Machetti, F.; Menchi, G.; Occhiato, E. G.; Scarpi, D.; Trabocchi, A. *Tetrahedron* **2002**, *58*, 9865–9870.
30. Trabocchi, A.; Cini, N.; Menchi, G.; Guarna, A. *Tetrahedron Lett.* **2003**, *44*, 3489–3492.
31. Campestre, C.; Agamennone, M.; Tortorella, P.; Prezioso, S.; Biasone, A.; Gavazzo, E.; Pochetti, G.; Mazza, F.; Hiller, O.; Tschesche, H.; Consalvi, V.; Gallina, C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 20–24.
32. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19*, 1639–1662.
33. Gasteiger, J.; Marsili, M. *Tetrahedron* **1980**, *36*, 3219–3228.
34. Banci, L.; Bertini, I.; Ciulli, A.; Fragai, M.; Luchinat, C.; Terni, B. *J. Mol. Catal. A: Chem.* **2003**, *204–205*, 401–408.
35. Sattler, M.; Schleucher, J.; Griesinger, C. *Prog. Nucl. Magn. Reson. Spectrosc.* **1999**, *34*, 93–158.
36. Leslie, A. G. W. In *Crystallographic Computing V*; Moras, D., Podjarny, A. D., Thierry, J. C., Eds.; Molecular Data Processing; Oxford University: Oxford, 1991; pp 50–61.
37. Evans, P. R. Data Reduction, Proceedings of CCP4 Study Weekend. Data Collection & Processing, 1993, pp 114–122.
38. Rossmann, M. G.; Blow, D. M. *Acta Crystallogr.* **1962**, *D 15*, 24–31.
39. Crowther, R. A. In *The Molecular Replacement Method*; Rossmann, M. G., Ed.; Gordon & Breach: New York, 1972.
40. Vagin, A.; Teplyakov, A. *J. Appl. Crystallogr.* **1997**, *30*, 1022–1025.
41. Vagin, A.; Teplyakov, A. *Acta Crystallogr.* **2000**, *D 56*, 1622–1624.
42. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr.* **1997**, *D 53*, 240–255.
43. McRee, D. E. *J. Mol. Graph.* **1992**, *10*, 44–47.
44. Lamzin, V. S.; Wilson, K. S. *Acta Crystallogr.* **1993**, *D 49*, 129–147.
45. Laskowski, R. A.; MacArthur, M. W.; Moss, D. S.; Thornton, J. M. *J. Appl. Crystallogr.* **1993**, *26*, 283–291.

Synthesis of a Bicyclic Proline Analogue from L-Ascorbic Acid

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Abstract: The efficient synthesis of a bicyclic α-amino acid from L-ascorbic acid is presented. The synthetic procedure is a three-step process involving an S_N2 reaction of an amino acetal with an L-ascorbic acid derivative, followed by protection of the amine as a Fmoc urethane, and acid-promoted *trans*-acetalization to give the title compound. Inversion of the configuration at the stereocenter of the precursor derived from L-ascorbic acid allowed the formation of the corresponding bicyclic α-amino acid bearing the carboxylic group in the 2-*exo* configuration. Such Fmoc-protected α-amino acids can be considered as bicyclic mimetics of proline, and are particularly suited for solid-phase peptidomimetic chemistry.

Key words: scaffold, peptidomimetic, amino acids, peptides, proline

During the last years, much interest has been paid to peptidomimetics, both in organic and medicinal chemistry, as they are much more selective and efficient than native peptides. Furthermore, they can have fewer side effects and greater oral bioavailability, as the lowered enzymatic degradation allows for longer biological activity.[1] Therefore, there is an ever-increasing need for versatile scaffolds, including new amino acid templates, to be applied in peptidomimetic design.[2] Among the various approaches for mimicking peptide structures,[3] numerous mimetics and analogues of proline have been developed and applied in the synthesis of biologically active compounds,[4] especially with the aim of modulating the *cis*/*trans* isomerism of acyl–proline bonds, and producing proline-like reverse turn inducers.[5]

Since the development of the first examples of 6,8-dioxa-3-azabicyclo[3.2.1]octane-based scaffolds (BTAa, see Figure 1), functionalities have been introduced at positions 3, 4, 5, and 7,[6] whereas position 2 has remained largely unexplored, occupied only by C=O, C=S, or CH_2 groups. In particular, the possibility of generating scaffolds with differently positioned carboxy groups (Figure 1, **A**–**C**) has been pursued recently, to expand the scope of peptidomimetic chemistry within this class of bicyclic scaffolds. For this approach, new synthetic strategies have been necessary, using different building blocks from the chiral pool.[7]

Recently, we moved from tartaric acid to sugars as building blocks for new versatile scaffolds in enantiopure form with complete control of the stereochemistry.[7] In particular, it was possible to generate new enantiopure bicyclic amino acids, such as γ- or δ-amino acids as reverse turn inducers, by use of erythrose derivatives[7b] and bicyclic proline mimetics starting from serine and glyceraldehyde derivatives.[7a] We reasoned that starting from L-ascorbic acid derivative **1** (Scheme 1), we could produce a new set of scaffolds bearing a substituent at position 2 (Figure 1, **D**). L-Ascorbic acid has appeared in literature as a valuable source of chiral building blocks for the preparation of enantiopure β-lactams[8] and L-hexoses.[9] Moreover, the use of such an inexpensive starting material is an advantage in multigram-scale organic synthesis.

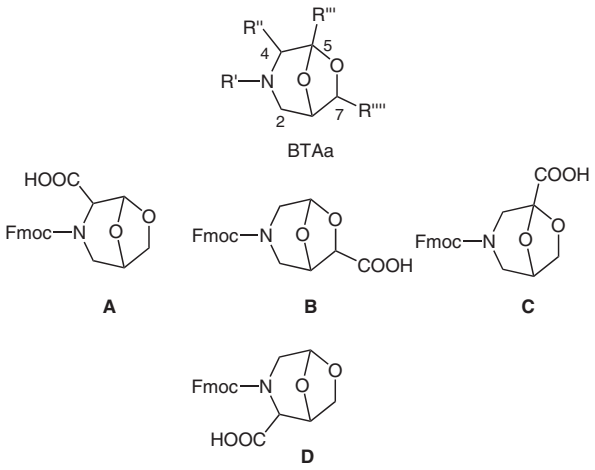

Figure 1 General formula of BTAa scaffolds (*top*) and isomeric bicyclic amino acids **A**–**D**, with **A** and **D** as proline mimetics

Thus, starting from triflate **2**, obtained from the protected L-ascorbic acid derivative **1**,[8,10] compound **4** was obtained in 99% yield by nucleophilic substitution with aminoacetaldehyde dimethyl acetal (**3**) at room temperature and after overnight stirring (Scheme 1). Further protection gave Fmoc urethane **5**, which was obtained with 9-fluorenylmethyl chloroformate in 1,4-dioxane as solvent, whereas *N*-(9-fluorenylmethoxycarbonyloxy)succinimide did not yield any product. Then, Fmoc derivative **5** was subjected to acid cyclization at 0 °C, according to reported procedures,[7] to afford the the methyl ester of scaffold **6**. Surprisingly, the corresponding carboxylic acid **6** was obtained as the major product by concomitant deprotection of the carbomethoxy group, and the conversion to acid **6** went to completion when the reaction was conducted at 25 °C. Since the preparation of 4-*endo*-carboxy scaf-

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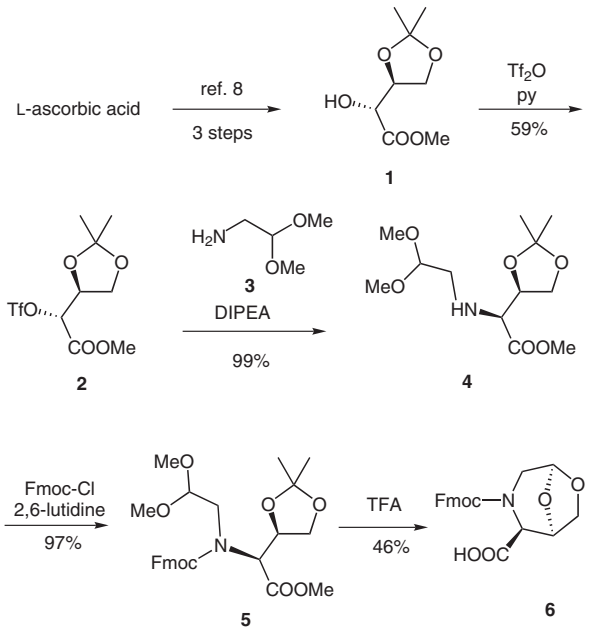
folds proved to be problematic and low yielding, as previously reported (see Figure 1, structure **A**),[7a] the facile synthesis of **6**, with the carboxy group in the *endo* position, provides a more complete collection of bicyclic amino acids for application in peptidomimetic chemistry.

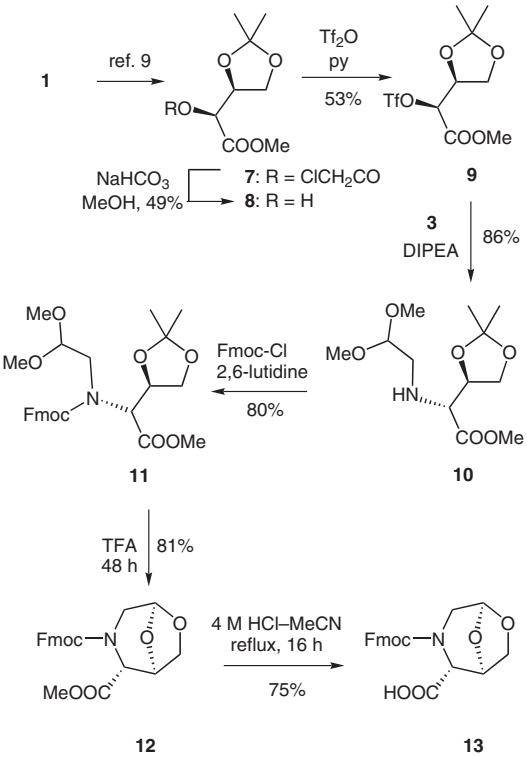


Scheme 1 Synthesis of a bicyclic α-amino acid with an *endo*-carboxy group from L-ascorbic acid

Formal inversion of the configuration at the C-2 stereocenter of compound **1** gives **13**, the corresponding diastereomer of **6**, carrying the carboxy group in the 2-*exo* position (Scheme 2). Thus, treatment of L-ascorbic acid derivative **1** with chloroacetic acid and triphenylphosphine, as previously reported,[9] produced the corresponding ester derivative **7** with inversion of configuration at C-2 (Scheme 2). Subsequent hydrolysis with sodium hydrogen carbonate in place of triethylamine gave **8**, the stereoisomer of **1**, which was converted into the corresponding triflate derivative **9** in 53% yield. The reaction of triflate **9** with acetal **3** gave adduct **10**, a diastereomer of **4**, with inversion of configuration at C-2. Successively, Fmoc protection by the same procedure used to prepare **5** yielded **11**, which was subsequently cyclized by treatment with trifluoroacetic acid. Interestingly, in this case, the reaction provided the bicyclic scaffold as methyl ester derivative **12** (Scheme 2), since the concomitant hydrolysis failed to occur, probably because of the axial orientation of the methoxycarbonyl group. This led to the hypothesis that the facile hydrolysis to give compound **6** might occur through the urethane carbonyl group providing anchimeric assistance to the equatorial methoxycarbonyl group. Compound **12** could be obtained in excellent yield when the cyclization time was prolonged from 16 hours (35% yield) to 48 hours (81% yield).

Hydrolysis of **12** (Scheme 2) proved to be problematic, and different methods were tried. Specifically, basic hydrolysis with lithium hydroxide did not yield α-amino acid **13** in significant amounts, and partial Fmoc-deprotection of **12** was observed. Hydrolysis with a dioxane–water system at room temperature for 48 hours gave **13** in 19% conversion, and a similar result (17%) was achieved when ester **12** was treated with 4 M aqueous hydrogen chloride in acetonitrile. However, when ester **12** was refluxed in the same aqueous hydrogen chloride–acetonitrile system for 16 hours, acid **13** was obtained in satisfactory yield (75%) (Scheme 2).

In conclusion, a new bicyclic α-amino acid was synthesized in a three-step procedure starting from an L-ascorbic acid derivative, producing amino acid derivative **6** directly after acid cyclization. In addition, inversion of configuration at the carbon atom bearing the triflate group of the L-ascorbic acid derivative allowed the synthesis of the corresponding diastereomeric bicyclic amino acid **13**, which has the carboxy group in the 2-*exo* configuration. These two new bicyclic proline analogues may thus find application in peptidomimetic research, and, in particular, are suited for solid-phase organic and peptide synthesis by the Fmoc protocol.



Scheme 2 Synthesis of a bicyclic α-amino acid with an *exo*-carboxy group from L-ascorbic acid

Melting points are uncorrected. Chromatographic separations were performed on silica gel by flash-column techniques. R_f values were obtained by TLC carried out on 25-mm silica gel plates (Merck F254), with the same eluent used for the column chromatography. ^{1}H and ^{13}C NMR spectra were recorded with a Varian Gemini 200 or a Varian MercuryPlus 400 instrument. IR spectra of CH_2Cl_2 or $CDCl_3$ solns were recorded on a Perkin-Elmer 881 spectrophotometer. Mass spectra were carried out with a Shimadzu spectrometer

operating with EI at 70 eV. Microanalyses were carried out with a Perkin-Elmer 2400/2 elemental analyzer. Optical rotations were determined on a JASCO DIP-370 instrument.

Methyl (*R*)-[(*S*)-2,2-Dimethyl-1,3-dioxolan-4-yl](trifluoromethylsulfonyloxy)acetate (2)

A soln of **1** (5.00 g, 26.3 mmol) in dry CH_2Cl_2 (45.5 mL) was cooled to –10 °C, and precooled dry pyridine (4.50 mL) was added. Then a soln of Tf_2O (7.30 mL, 34.2 mmol) in dry CH_2Cl_2 (13.6 mL) was added over 30 min, and the mixture was stirred at r.t. for 30 min. After the organic phase had been washed with a sat. $NaHCO_3$ soln (3 × 50 mL), the organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo to give a dark oil. Flash chromatography (silica gel, PE–EtOAc, 2:1) afforded **2** as a white solid; yield: 4.98 g (59%).

Mp 51–54 °C; $[\alpha]_D^{25}$ +38.8 (*c* 0.8, CH_2Cl_2); R_f = 0.6 (PE–EtOAc, 2:1).

IR ($CDCl_3$): 3052, 2986, 1733, 1265 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 5.04 (d, *J* = 5.5 Hz, 1 H, TfOCH), 4.56–4.52 (m, 1 H, ring H-4), 4.18 (dd, *J* = 9.4, 6.7 Hz, 1 H, C*H*H), 4.06 (dd, *J* = 9.4, 4.7 Hz, 1 H, CH*H*), 3.88 (s, 3 H, OCH_3), 1.45 (s, 3 H, CH_3), 1.36 (s, 3 H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.1 (s, C=O), 121.5 (s, CF_3), 111.1 [s, $C(CH_3)_2$], 82.6 (d, TfOCH), 74.1 (d, ring C-4), 65.4 (t, CH_2), 53.5 (q, OCH_3), 25.9 (q, CH_3), 25.0 (q, CH_3).

MS (EI, 70 eV): *m*/*z* (%) = 322 (8) [M^+], 69 (100), 55 (77).

Anal. Calcd for $C_9H_{13}F_3O_7S$: C, 33.54; H, 4.07. Found: C, 33.46; H, 3.99.

Methyl (*S*)-[(2,2-Dimethoxyethyl)amino][(*R*)-2,2-dimethyl-1,3-dioxolan-4-yl]acetate (4)

A soln of **2** (1.30 g, 4.01 mmol) in dry CH_2Cl_2 (20 mL) was cooled to 0 °C under N_2, and then a soln of **3** (0.50 mL, 4.81 mmol) and DIPEA (1.40 mL, 8.02 mmol) in dry CH_2Cl_2 (20 mL) was added. The mixture was stirred at r.t. for 15 h, and then it was extracted with a sat. $NaHCO_3$ soln (3 × 40 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo to give a dark oil. Flash chromatography (silica gel, PE–EtOAc, 3:1) afforded pure **4** as a yellow oil; yield: 1.11 g (99%).

$[\alpha]_D^{25}$ +15.6 (*c* 0.9, CH_2Cl_2); R_f = 0.47 (PE–EtOAc, 3:1).

IR ($CDCl_3$): 2991, 2955, 2836, 1733, 1250, 1219 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 4.41 [t, *J* = 4.8 Hz, 1 H, $(OCH_3)_2CHCH_2NH$)], 4.18–4.13 (m, 1 H, ring H-4), 4.07–4.00 (m, 2 H, ring CH_2), 3.76 (s, 3 H, CO_2CH_3), 3.35 (s, 3 H, OCH_3), 3.34 (s, 3 H, OCH_3), 3.27 (d, *J* = 7.3 Hz, 1 H, C*H*NH), 2.76 (dd, *J* = 12.1, 6.3 Hz, 1 H, CH_2NH), 2.63 (dd, *J* = 12.1, 4.6 Hz, 1 H, CH_2NH), 1.72 (br, 1 H, NH), 1.41 (s, 3 H, CH_3), 1.31 (s, 3 H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.3 (s, C=O), 109.7 (s), 103.5 [d, *C*H$(OMe)_2$], 76.8 [d, *C*HOC$(CH_3)_2$], 67.1 (t, CH_2NH), 64.4 (d, *C*HCO_2Me), 53.9 (q, OCH_3), 53.2 (q, OCH_3), 51.9 (q, OCH_3), 49.5 (t, ring CH_2), 26.7 (q, CH_3), 25.3 (q, CH_3).

MS (EI, 70 eV): *m*/*z* (%) = 277 (4) [M^+], 177 (79), 144 (100), 75 (96).

Anal. Calcd for $C_{12}H_{23}NO_6$: C, 51.97; H, 8.36; N, 5.05. Found: C, 51.84; H, 8.40; N, 5.12.

Methyl (*S*)-[(2,2-Dimethoxyethyl)(9*H*-fluoren-9-ylmethoxycarbonyl)amino][(*R*)-2,2-dimethyl-1,3-dioxolan-4-yl]acetate (5)

To a soln of **4** (616 mg, 2.22 mmol) in dioxane (44 mL), at 0 °C and under N_2 were added FmocCl (863 mg, 3.33 mmol) and 2,6-lutidine (388 μL, 3.33 mmol). The mixture was stirred at r.t. for 15 h. The soln was then concentrated in vacuo, the crude was dissolved in CH_2Cl_2 (20 mL), and the soln was washed with 5% citric acid soln (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo to give **5** as a clear oil. Flash chromatography (silica gel, PE–EtOAc, 5:1) afforded **5** as a colorless oil; yield: 1.07 g (97%).

$[\alpha]_D^{25}$ –48.1 (*c* 1.45, CH_2Cl_2); R_f = 0.1 (PE–EtOAc, 5:1).

IR ($CDCl_3$): 2958, 1794, 1709 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 7.68 (d, *J* = 5.4 Hz, 2 H, Ar), 7.51 (d, *J* = 3.9 Hz, 2 H, Ar), 7.33–7.22 (m, 4 H, Ar), 4.61 (m, 2 H), 3.62 (s, 3 H, OCH_3), 4.56–3.02 (m, 14 H), 1.28 (s, 3 H, CH_3), 1.25 (s, 3 H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) (mixture of rotamers): δ = 169.7 (s), 156.1 and 155.9 (s), 143.5 (s, 2 C), 141.4 and 141.3 (s, 2 C), 127.7 (d, 2 C), 127.1 (d, 2 C), 124.8 and 124.5 (d, 2 C), 120.1 and 119.9 (d, 2 C), 110.1 and 109.9 (s), 103.4 and 102.9 (d), 74.9 and 74.6 (d), 67.2 and 66.6 (d), 66.1 (q), 62.2 and 61.9 (d), 55.3 and 55.1 (t), 54.4 and 54.1 (t), 52.2 (q, 2 C), 49.6 (d), 47.4 and 47.1 (t), 26.8 and 25.4 (q), 26.6 and 25.4 (q).

MS (EI, 70 eV): *m*/*z* (%) = 499 (0.3) [M^+], 178 (99), 75 (100).

Anal. Calcd for $C_{27}H_{33}NO_8$: C, 64.92; H, 6.66; N, 2.80. Found: C, 64.91; H, 6.62; N, 2.75.

(1*R*,2*S*,5*S*)-3-(9-Fluorenylmethoxycarbonyl)-6,8-dioxa-3-azabicyclo[3.2.1]octane-2-*endo*-carboxylic Acid (6)

Compound **5** (1.02 g, 2.00 mmol) was dissolved in TFA (4.20 mL), and the soln was stirred at 25 °C overnight. The soln was then concentrated in vacuo to give a dark oil. Flash chromatography (silica gel, CH_2Cl_2–MeOH, 20:1, buffered with 0.1% TFA) afforded **6** as a white solid; yield: 0.351 g (46%).

Mp = 198–201 °C; $[\alpha]_D^{25}$ –78.1 (*c* 1.0, CH_2Cl_2–1% TFA); R_f = 0.3 (CH_2Cl_2–MeOH, 20:1, buffered with 0.1% TFA).

IR ($CDCl_3$): 3691, 2958, 1794, 1602 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$) (2:1 mixture of rotamers): δ = 7.69 (d, *J* = 7.1 Hz, 2 H, Ar), 7.50 (d, *J* = 7.8 Hz, 2 H, Ar), 7.33–7.22 (m, 4 H, Ar), 5.16 (s, 2/3 H, H-5, rot. A), 5.11 (d, *J* = 2.7 Hz, 1/3 H, H-5, rot. B), 4.68–4.21 (m, 7 H), 3.95 (d, *J* = 13.4 Hz, 1/3 H, H-4, rot. B), 3.85 (d, *J* = 13.6 Hz, 2/3 H, H-4, rot. A), 3.06 (d, *J* = 13.6 Hz, 2/3 H, H-4, rot. A), 2.99 (d, *J* = 13.4 Hz, 1/3 H, H-4, rot. B).

^{13}C NMR (100 MHz, $CDCl_3$) (mixture of rotamers): δ = 173.0 (s, O–C=O), 158.6 (s, N–C=O), 143.2 (s, 2 C, Ar), 141.0 (s, 2 C, Ar), 127.9 (d, 2 C, Ar), 127.4 and 127.3 (d, 2 C, Ar), 124.9 (d, 2 C, Ar), 120.1 and 119.8 (d, 2 C, Ar), 88.7 and 88.6 (d, C-5), 72.2 and 71.2 (t, C-7), 70.9 and 69.8 (t), 65.4 and 65.0 (d, C-1), 53.9 and 53.8 (d, C-2), 47.0 and 46.7 (d), 44.4 and 43.7 (t, C-4).

MS (EI, 70 eV): *m*/*z* (%) = 381 (1.3) [M^+], 178 (100).

Anal. Calcd for $C_{21}H_{19}NO_6$: C, 66.13; H, 5.02; N, 3.67. Found: C, 65.94; H, 4.94; N, 3.55.

Methyl (*S*)-[(*S*)-2,2-Dimethyl-1,3-dioxolan-4-yl](trifluoromethylsulfonyloxy)acetate (9)

A soln of **8** (150 mg, 0.79 mmol) in dry CH_2Cl_2 (1.4 mL) was cooled to –10 °C, precooled dry pyridine (135 μL) was added, followed by the addition of a soln of Tf_2O (219 μL, 1.02 mmol) in dry CH_2Cl_2 (0.40 mL) over 30 min. The mixture was stirred at r.t. for 30 min and then neutralized with a sat. $NaHCO_3$ soln. The organic layer was separated, dried (Na_2SO_4), filtered, and concentrated in vacuo to give a dark oil. Flash chromatography (silica gel, PE–EtOAc, 2:1) afforded **9** as a yellow oil; yield: 136 mg (53%).

R_f = 0.6 (PE–EtOAc, 2:1).

^1H NMR (200 MHz, $CDCl_3$): δ = 5.24 (d, *J* = 3.6 Hz, 1 H, TfOCH), 4.58–4.50 (m, 1 H, ring H-4), 4.10–3.94 (m, 2 H, ring CH_2), 3.84 (s, 3 H, OCH_3), 1.43 (s, 3 H, CH_3), 1.34 (s, 3 H, CH_3).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 165.0 (s, C=O), 121.5 (s, CF_3), 110.9 [s, $C(CH_3)_2$], 81.0 (d, TfOCH), 74.0 (d, ring H-4), 65.4 (t, CH_2), 53.5 (q, O CH_3), 25.8 (q, OCH_3), 24.9 (q, CH_3).

MS (EI, 70 eV): *m*/*z* (%) = 322 (3) [M$^+$], 75 (100), 55 (62).

Methyl (*R*)-[(2,2-Dimethoxyethyl)amino][(*R*)-2,2-dimethyl-1,3-dioxolan-4-yl]acetate (10)

A soln of **9** (136 mg, 0.42 mmol) in dry CH_2Cl_2 (2.1 mL) was cooled to 0 °C under N_2, and then a soln of **3** (56 μL, 0.51 mmol) and DIPEA (144 μL, 0.84 mmol) in dry CH_2Cl_2 (2.1 mL) was added. The mixture was stirred under N_2 at r.t. overnight, and then neutralized with a soln of $NaHCO_3$. The organic layer was separated, dried (Na_2SO_4), filtered, and concentrated in vacuo to give a dark oil. Flash chromatography (silica gel, PE–EtOAc, 3:1) afforded pure **10** as a colorless oil; yield: 101 mg (86%).

$[\alpha]_D^{25}$ +19.3 (*c* 0.7, CH_2Cl_2); R_f = 0.47 (PE–EtOAc, 3:1).

IR (CH_2Cl_2): 2990, 2954, 2834, 1739, 1271, 1261 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 4.41 [t, *J* = 5.4 Hz, 1 H, $(CH_3O)_2CHCH_2NH$], 4.26 (q, *J* = 6.5 Hz, 1 H, ring H-4), 4.01–3.86 (m, 2 H, ring CH_2), 3.71 (s, 3 H, OCH_3 ester), 3.31 (s, 7 H, OCH_3, $CHNH$), 2.80 (dd, *J* = 12.1, 5.9 Hz, 1 H, CH_2NH), 2.57 (dd, *J* = 12.4, 5.1 Hz, 1 H, CH_2NH), 1.36 (s, 3 H, CH_3), 1.29 (s, 3 H, CH_3).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.7 (s, C=O), 109.6 (s, C_{quat}), 103.9 [d, $CH(OCH_3)_2$], 76.2 [d, $CHOC(CH_3)_2$], 66.4 (t, CH_2NH), 62.9 (d, $CHCO_2Me$), 54.0 (q, OCH_3), 53.2 (q, OCH_3), 52.1 (q, OCH_3), 49.5 (t, ring CH_2), 26.5 (q, CH_3), 25.4 (q, CH_3).

MS (EI, 70 eV): *m*/*z* (%) = 277 (6) [M$^+$], 177 (75), 144 (89), 75 (100).

Anal. Calcd for $C_{12}H_{23}NO_6$: C, 51.97; H, 8.36; N, 5.05. Found: C, 52.10; H, 8.55; N, 5.14.

Methyl (*R*)-[(2,2-Dimethoxyethyl)(9H-fluoren-9-ylmethoxycarbonyl)amino][(*R*)-2,2-dimethyl-1,3-dioxolan-4-yl]acetate (11)

To soln of **10** (80 mg, 0.29 mmol) in dioxane (5.8 mL), at 0 °C and under N_2, was added FmocCl (112 mg, 0.43 mmol) and 2,6-lutidine (50 μL, 0.43 mmol). The mixture was stirred at r.t. under N_2 for 15 h. The solvent was concentrated in vacuo, then the crude was dissolved in CH_2Cl_2, and the soln was washed with 5% citric acid soln (3 × 10 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo to give a clear oil. Flash chromatography (silica gel, PE–EtOAc, 5:1) afforded **11** as a colorless oil; yield: 115 g (80%).

$[\alpha]_D^{25}$ +48.4 (*c* 0.95, CH_2Cl_2); R_f = 0.1 (PE–EtOAc, 5:1).

IR (CH_2Cl_2): 2988, 1739, 1701, 1261, 1066 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$) (mixture of rotamers): δ = 7.81–7.74 (m, 2 H, Ar), 7.63–7.53 (m, 2 H, Ar), 7.44–7.31 (m, 4 H, Ar), 4.77–3.55 (m, 10 H), 3.68 (s, 3 H, OCH_3), 3.37 and 3.32 (s, 6 H, OCH_3), 1.41 (s, 3 H, CH_3), 1.36 (s, 3 H, CH_3).

^{13}C NMR (50 MHz, $CDCl_3$) (mixture of rotamers): δ = 169.4 and 168.7 (s), 155.5 and 155.3 (s), 143.7 and 143.6 (s, 2 C), 141.6 and 141.3 (s, 2 C), 127.6 and 127.0 (d, 2 C), 124.8 (d, 2 C), 124.3 and 124.1 (d, 2 C), 120.1 and 119.9 (d, 2 C), 108.9 and 108.6 (s), 104.4 and 104.3 (d), 73.6 and 73.5 (d), 68.6 and 68.5 (d), 67.1 (q), 65.0 and 64.5 (d), 55.8 and 55.6 (t), 55.5 and 55.4 (t), 52.3 and 51.9 (q), 51.8 and 51.3 (q), 47.4 and 47.1 (t), 26.8 and 26.7 (q), 25.2 and 25.1 (q).

MS (EI, 70 eV): *m*/*z* (%) = 499 (0.3) [M$^+$], 178 (100), 75 (60).

Anal. Calcd for $C_{27}H_{33}NO_8$: C, 64.92; H, 6.66; N, 2.80. Found: C, 64.90; H, 6.51; N, 2.70.

Methyl (1*R*,2*R*,5*S*)-3-(9-Fluorenylmethoxycarbonyl)-6,8-dioxa-3-azabicyclo[3.2.1]octane-2-*exo*-carboxylate (12)

Compound **11** (226 mg, 0.45 mmol) was dissolved in TFA (950 μL), and the soln was stirred at 25 °C for 48 h. Then the soln was concentrated in vacuo; this gave a dark oil, which was purified by flash chromatography (silica gel, CH_2Cl_2–MeOH, 20:1, buffered with 0.1% TFA) to give pure **12** as a white solid; yield: 145 mg (81%).

Mp 56–59 °C; $[\alpha]_D^{25}$ +48.5 (*c* 0.35, CH_2Cl_2); R_f = 0.8 (CH_2Cl_2–MeOH, 20:1, buffered with 0.1% TFA).

IR (CH_2Cl_2): 2927, 1752, 1708, 1269 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$) (3:2 mixture of rotamers): δ = 7.69 (m, 2 H, Ar), 7.51 and 7.41 (m, 2 H, Ar), 7.36–7.21 (m, 4 H, Ar), 5.45 (s, 1 H, H-5), 4.92 (d, *J* = 3.9 Hz, 3/5 H, H-1, rot. A), 4.78 (d, *J* = 3.9 Hz, 2/5 H, H-1, rot. B), 4.52–3.64 (m, 7 H), 3.74 and 3.64 (s, 3 H, OCH_3), 3.30 (d, *J* = 12.5 Hz, 3/5 H, H-4, rot. A), 3.14 (d, *J* = 13.0 Hz, 2/5 H, H-4, rot. B).

^{13}C NMR (100 MHz, $CDCl_3$) (mixture of rotamers): δ = 168.9 (s, O–C=O), 156.6 (s, N–C=O), 143.5 (s, 2 C, Ar), 141.2 (s, 2 C, Ar), 127.7 and 127.0 (d, 2 C, Ar), 124.9 (d, 2 C, Ar), 124.6 and 124.5 (d, 2 C, Ar), 120.0 (d, 2 C, Ar), 98.9 and 98.4 (d, C-5), 72.7 and 72.3 (d, C-1), 68.2 and 67.7 (t), 67.3 (t, C-7), 59.5 and 58.9 (d, C-2), 52.8 (q), 47.5 and 47.2 (t, C-4), 47.3 (d).

MS (EI, 70 eV): *m*/*z* (%) = 395 (0.7) [M$^+$], 336 (2), 178 (100), 165 (14), 89 (7), 55(8).

Anal. Calcd for $C_{22}H_{21}NO_6$: C, 66.83; H, 5.35; N, 3.54. Found: C, 66.34; H, 5.30; N, 3.46.

(1*R*,2*R*,5*S*)-3-(9-Fluorenylmethoxycarbonyl)-6,8-dioxa-3-azabicyclo[3.2.1]octane-2-*exo*-carboxylic Acid (13)

A soln of **12** (55 mg, 0.14 mmol) in MeCN (2 mL) and 4 M HCl (3 mL) was refluxed for 16 h, and then the solvent was evaporated in vacuo. The white solid was treated with Et_2O (10 mL), and the soln was filtered and evaporated. This gave a yellow solid, which was purified by flash chromatography (silica gel, CH_2Cl_2–MeOH, 20:1, buffered with 0.1% TFA), to give **13** as a white solid; yield: 40 mg (75%).

Mp 86–88 °C; $[\alpha]_D^{25}$ +62.9 (c 0.5, CH_2Cl_2); R_f = 0.2 (CH_2Cl_2–MeOH, 20:1, buffered with 0.1% TFA).

IR ($CDCl_3$): 3066, 2960, 2900, 1709, 1451, 1413 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$) (3:2 mixture of rotamers): δ = 10.34 (br, 1 H, COOH), 7.75 (m, 2 H, Ar), 7.60–7.27 (m, 6 H, Ar), 5.55 and 5.53 (s, 1 H, H-5), 5.04 (d, *J* = 4.6 Hz, 3/5 H, H-1, rot. A), 4.86 (m, 2/5 H, H-1, rot. B), 4.66–3.67 (m, 7 H), 3.36 (d, *J* = 12.8 Hz, 3/5 H, H-4, rot. A), 3.19 (d, *J* = 13.0 Hz, 2/5 H, H-4, rot. B).

^{13}C NMR (50 MHz, $CDCl_3$) (mixture of rotamers): δ = 173.5 and 173.3 (s, O–C=O), 156.5 and 155.4 (s, N–C=O), 143.2 (s, 2 C, Ar), 140.9 (s, 2 C, Ar), 128.4 (d, 2 C, Ar), 127.5 (d, 2 C, Ar), 124.7 and 124.3 (d, 2 C, Ar), 119.7 (d, 2 C, Ar), 98.6 and 98.0 (d, C-5), 72.3 and 71.8 (d, C-1), 67.9 (t), 67.3 and 66.9 (t, C-7), 58.7 and 58.4 (d, C-2), 47.1 and 46.7 (t, C-4), 46.8 (d).

MS (EI, 70 eV): *m*/*z* (%) = 381 (0.3) [M$^+$], 178 (100).

Anal. Calcd for $C_{21}H_{19}NO_6$: C, 66.13; H, 5.02; N, 3.67. Found: C, 66.05; H, 4.92; N, 3.65.

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References

(1) (a) Giannis, A.; Kolter, T. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1244. (b) Gante, J. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1699.

(2) Trabocchi, A.; Guarna, F.; Guarna, A. *Curr. Org. Chem.* **2005**, *9*, 1127.

(3) See for example: Hanessian, S.; McNaughton-Smith, G.; Lombart, H.-G.; Lubell, W. D. *Tetrahedron* **1997**, *53*, 12789.

(4) (a) Damour, D.; Herman, F.; Labaudinière, R.; Pantel, G.; Vuilhorgne, M.; Mignani, S. *Tetrahedron* **1999**, *55*, 10135. (b) Wang, Q.; Tran Huu Dau, M.-E.; Sasaki, N. A.; Potier, P. *Tetrahedron* **2001**, *57*, 6455. (c) Demange, L.; Cluzeau, J.; Ménez, A.; Dugave, C. *Tetrahedron Lett.* **2001**, *42*, 651. (d) Gorichko, M. V.; Grygorenko, O. O.; Komarov, I. V. *Tetrahedron Lett.* **2002**, *43*, 9411. (e) Dahlgren, A.; Bråualt, J.; Kuaenström, I.; Nilsson, I.; Musil, D.; Samuelsson, B. *Bioorg. Med. Chem.* **2002**, *10*, 1567. (f) Wang, X. J.; Hart, S. A.; Xu, B.; Mason, M. D.; Goodell, J. R.; Etzkorn, F. A. *J. Org. Chem.* **2003**, *68*, 2343. (g) Donkor, I. O.; Korukonda, R.; Huang, T. L.; Le Cour, L. Jr. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 783. (h) Jeannotte, G.; Lubell, W. D. *J. Org. Chem.* **2004**, *69*, 4656. (i) Liu, Y.-T.; Wong, J. K.; Tao, M.; Osterman, R.; Sannigrahi, M.; Girijavallabhan, V. M.; Saksena, A. K. *Tetrahedron Lett.* **2004**, *45*, 6097.

(5) (a) Kym, K.; Germanas, J. P. *J. Org. Chem.* **1997**, *62*, 2847. (b) Halab, L.; Lubell, W. D. *J. Org. Chem.* **1999**, *64*, 3312. (c) Halab, L.; Lubell, W. D. *J. Am. Chem. Soc.* **2002**, *124*, 2474. (d) Chakraborty, T. K.; Ghosh, A.; Kumar, S. K.; Kunwar, A. C. *J. Org. Chem.* **2003**, *68*, 6459. (e) Zhang, W.-J.; Berglund, A.; Kao, J. L.-F.; Couty, J.-P.; Gershengorn, M. C.; Marshall, G. R. *J. Am. Chem. Soc.* **2003**, *125*, 1221. (f) Chierici, S.; Jourdan, M.; Figuet, M.; Dumy, P. *Org. Biomol. Chem.* **2004**, *2*, 2437.

(6) For an account, see: Trabocchi, A.; Menchi, G.; Guarna, F.; Machetti, F.; Scarpi, D.; Guarna, A. *Synlett* **2006**, 331.

(7) (a) Trabocchi, A.; Cini, N.; Menchi, G.; Guarna, A. *Tetrahedron Lett.* **2003**, *44*, 3489. (b) Trabocchi, A.; Menchi, G.; Rolla, M.; Machetti, F.; Bucelli, I.; Guarna, A. *Tetrahedron* **2003**, *59*, 5251. (c) Danieli, E.; Trabocchi, A.; Menchi, G.; Guarna, A. *Eur. J. Org. Chem.* **2005**, 4372.

(8) Wei, C. C.; De Bernardo, S.; Tengi, J. P.; Borgese, J.; Weigele, M. *J. Org. Chem.* **1985**, *50*, 3462.

(9) Ermolenko, L.; Sasaki, A. *J. Org. Chem.* **2006**, *71*, 693.

(10) Tanaka, A.; Yamashita, K. *Synthesis* **1987**, 570.